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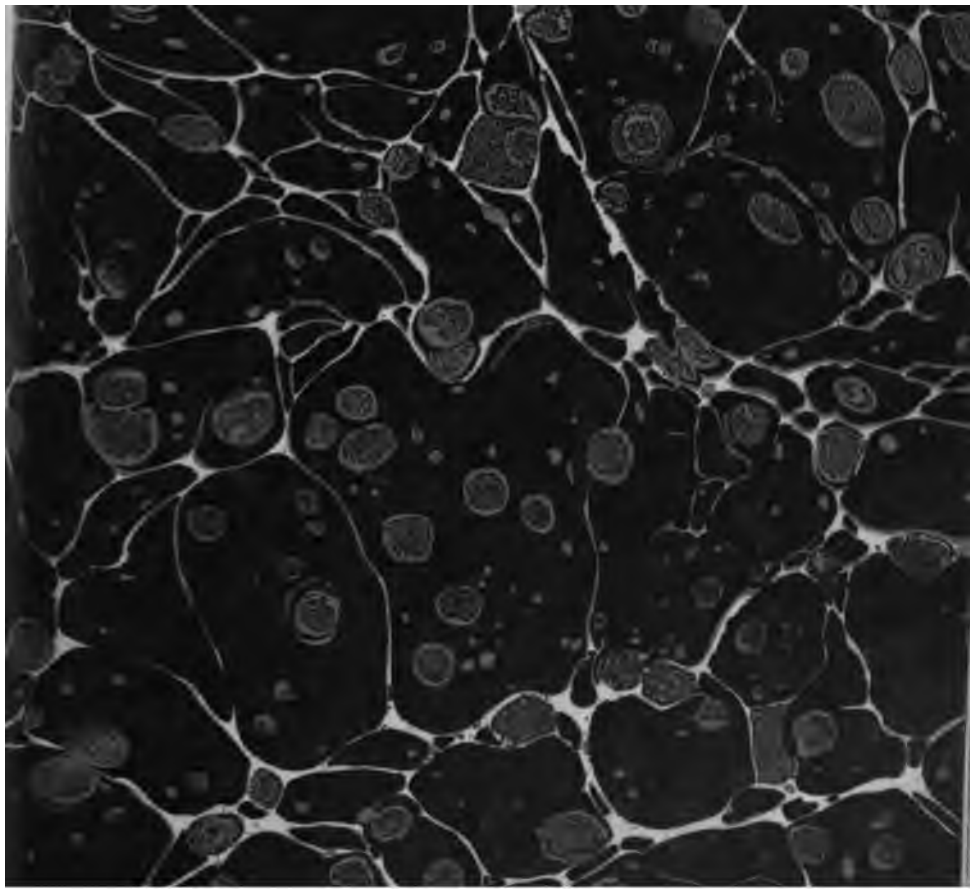
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**PROCEEDINGS OF THE AMERICAN SOCIETY OF  
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**PROCEEDINGS OF THE AMERICAN SOCIETY OF  
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**THE INFLUENCE OF FOOD ON METABOLISM.**

**By GRAHAM LUSK.**

In 1881 Voit laid down the principle that the intensity of metabolism in the cells was modified by the quality and quantity of the food materials brought to them by the blood. He believed that the inherent power of the cells to metabolize was augmented by the presence of increased quantities of foodstuffs. Rubner developed another conception. He declared that the fundamental metabolism of a normal warm-blooded animal was always constant and that the effect of food ingestion did not change this. The increased heat production which followed the taking of food was due to heat developed from a lot of intermediary reactions and oxidations and had nothing whatever to do with the fundamental level of the cellular requirement of energy which was entirely unchanged. Thus, when protein was metabolized it could supply energy for the maintenance of true cellular activity in so far as glucose was produced from it, whereas other intermediary cleavage products were simply oxidized with the production of extra heat, which was in no way involved in the life processes of the cells. The utilization of energy in protein might be compared with the burning of a tree as fuel for the steam engine, the trunk of the tree being used as fuel within the engine for the production of power, whereas the limbs and twigs are burned as brush outside and supply only heat.

In 1907 when I saw Voit just a few months before his death, in the seventy-seventh year of his age, he expressed his strong dissent from Rubner's views. At that time Rubner's theory had achieved wide acceptance, and the then known facts seemed to accord with his arguments.

The theory of Zuntz that the specific dynamic action of the foodstuffs is chiefly due to intestinal activity no longer merits serious discussion.

If one gives meat in large quantity to a dog, the heat production may be nearly doubled. During the second hour it reaches almost its maximal height. The third hour registers the highest metabolism, and the heat production remains at this level for nearly twelve hours, during which the nitrogen in the urine manifests a high and almost even output from hour to hour. The metabolism then gradually falls and nearly reaches the basal level about the twenty-first hour. During the hours of the high metabolism the heat value of the protein destroyed as calculated from the nitrogen in the urine is far in excess of the heat eliminated by the dog. This is due to the fact that part of the carbon-containing radicles derived from the protein destroyed is retained in the organism. This retained carbon might be deposited as fat or as glucose. That it is retained in the form of glucose is determined on the basis of the oxygen consumption and the computed heat value of the material retained. If it be assumed that the carbon is retained as glucose, the quantity of oxygen absorbed fits with the theory. If it were retained as fat, 10 per cent less oxygen would have been required than was actually found. Calculated on the basis that the carbon is retained as glucose, indirect and direct calorimetry agree closely. Twenty per cent of the energy value of protein was found to be thus capable of retention in the form of glucose. Erwin Voit at one time put forward the theory that the specific dynamic action of protein was due to the conversion of protein into fat. The above experiment disproves this idea. It will be shown later that the conversion of glucose into fat involves little energy change, so that even though over-ingestion of protein should be pushed so far as to cause the synthetic production of fat from protein, this event would not noticeably affect the heat production.

Experiments were instituted with the intention of more fully establishing the truth of Rubner's theories of specific dynamic action. It was known that glycocoll and alanine were completely convertible into glucose in the diabetic organism, whereas glutamic acid was in part so converted, three of its five carbon atoms passing into glucose, the other two being oxidized. It follows

from Rubner's hypothesis that glycocoll and alanine should exert no specific dynamic action, whereas glutamic acid should manifest this phenomenon. The reverse proved to be true; glycocoll and alanine are capable of greatly increasing the heat production, whereas the strong di-basic glutamic acid is without influence. Glycocoll and alanine produce powerful effects lasting eight and five hours respectively, whereas on giving those quantities of glucose into which the amino-acids are convertible an almost negligible influence is observed.

The increase in metabolism after giving glycocoll and alanine together is equal to the sum of the effects produced by either alone. Furthermore, the increase of metabolism after giving 20 grams of glycocoll is twice as great as after giving 10 grams. Similar relations obtain after giving different quantities of alanine. This accords with Rubner's discovery that the intensity of the specific dynamic action is proportional to the quantity of protein ingested. When one compares the heat-increasing power of glycocoll and alanine upon metabolism, it is found that this power is not proportional to their respective abilities to form sugar, but rather to the number of molecules of glycollic and lactic acids which they are respectively supposed to yield on deamination.

It was found in one experiment that the entire energy content of the ingested glycocoll reappeared in the extra output of energy given off by the dog in the form of heat. The course of inquiry into this phenomenon which naturally suggests itself is whether glycocoll is without action upon the body cells; that is, whether it merely explodes and yields heat, or whether it directly stimulates the cells thereby raising metabolism to a higher level. This point was determined by giving glycocoll to a phlorhizinized animal. Under these circumstances there is no oxidation of the material ingested and the energy content of the glycocoll is eliminated in the urine in the form of sugar and urea. The metabolism was largely increased, notwithstanding the fact that there was no oxidation of the ingested material. Exactly the same phenomenon followed the ingestion of alanine in phlorhizin glycosuria. The ingestion of glucose was without effect even after giving 70 grams. The cause of the specific dynamic action of glycocoll and alanine therefore lies in a chemical stimulation of

the cells causing them to metabolize more material. This confirms the older view of Voit that the action of food increases the power of the cells to metabolize.

The chemical stimulus to the cells does not reside in the amino-acids themselves, for (1) when meat is given and amino-acids are retained in the body for the synthesis of new protein there is no specific dynamic action, (2) there is also no accumulation of amino-acids in the tissues under these circumstances, and (3) the hours of high metabolism after giving glycocoll and alanine are the hours of the maximal metabolism of these amino-acids. From such facts it is obvious that metabolism products of amino-acids like glycollic and lactic acids are indicated as the substances which are the chemical stimuli. One recalls in this connection the permanently increased metabolism in phosphorus poisoning, in severe anemias, and in persons living at high altitudes, under all of which conditions lactic acid is found in increased amounts in the blood and often in the urine.

That the chemical stimulus acts on protoplasm directly and not through excitation of the nervous system is to be inferred from the experiments of O. Frank and F. Voit who noticed a large increase in the heat production of curarized dogs after giving them meat.

External cold acts reflexly through the nervous system to increase metabolism in a fasting animal and thus prevents a fall in body temperature. Rubner has called this the "chemical regulation" of body temperature. According to his hypothesis the "free heat" liberated in the intermediary metabolism of protein can be used in lieu of that derived from the increased metabolism induced through the effect of cold. In the light of the newer researches, however, the extra heat necessary to preserve the body from a fall in temperature may be directly derived from an increased metabolism of the cell itself, whether this be induced by nerve action or by direct chemical stimulation.

It has been found that ingested leucine and tyrosine also increase metabolism, though to a lesser extent than do glycocoll and alanine.

It may be that the mass action of the various fragments produced in the breakdown of protein in metabolism is also a contributory factor in the higher production of heat, but that it is the



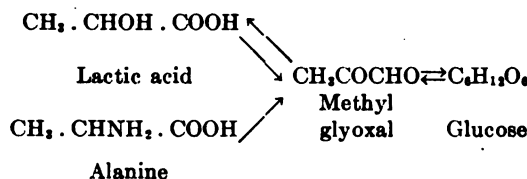
main factor is negated by contrasting the different effect of 20 grams of glutamic acid with that of 20 grams of glycocoll, the effect of the first being *nil* and that of the latter powerful.

Passing now to the consideration of the metabolism as influenced by the ingestion of fat and carbohydrates, it is found that an increase occurs whenever either fat or sugar is given.) Fat pours into the blood stream through the thoracic duct, and accompanying this increase in the food supply to the cells the metabolism rises. The behavior of sugars has especially been studied. The ingestion of 50 grams of glucose by a dog causes at first an increase in the percentage quantity of blood sugar, the metabolism rises and is maintained at a high level during the three or four hours usually required for the absorption of the material. At the end of the second hour the blood sugar has fallen to the normal percentage quantity, but the blood has become much more dilute. Little urine is secreted until the last hour of the high metabolism; during that hour a large volume of urine is eliminated, and during the following hour the original basal metabolism is reached. During the period of high metabolism the respiratory quotient approximates unity, whereas subsequently this quotient may fall to a level which indicates the oxidation of a mixture of carbohydrate and fat. With the cessation of absorption the influx of glucose molecules which were carried by an increased volume of blood ceases, their effect on the cells is cut off, and the metabolism falls. After giving 70 grams of glucose the heat production may be 35 per cent above the basal metabolism during the hours of increased metabolism. All these details are obscured in long experiments lasting twenty-four hours. (It seems as though the quantity of fuel which nourished the cells determined the height of metabolism in the older sense of Voit. That the change in metabolism is not due to altered osmotic relations is evidenced by the fact that solutions of urea or common salt when ingested by a dog have no influence on his metabolism.)

For a more complete understanding of the probable action of carbohydrates one must consider their intermediary metabolism.

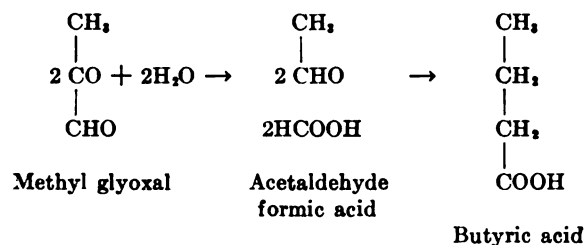
Dakin has shown how methyl glyoxal may be obtained from both lactic acid and alanine; how it may also be formed *in vitro* from glucose, and be converted into glucose if it be given in phlorhizin glycosuria. He gives the name glyoxalases to those tissue enzymes which rapidly convert methyl glyoxal into lactic acid.

The reactions which probably occur in the organism may be written as follows:

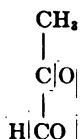


The formation of methyl glyoxal as an intermediary product of carbohydrate metabolism explains the synthetic production of glucose from fructose in the organism. For if fructose be first converted into methyl glyoxal radicles which are free from asymmetric carbon atoms, the latter can then be synthesized into glucose, the sugar of the body.

It has been affirmed by Woodyatt that lactic acid is not produced from carbohydrate in the organism except in the cases of oxygen insufficiency. The first cleavage products of methyl glyoxal may then be acetaldehyde and formic acid.



In the course of the usual metabolism of carbohydrate these substances would be oxidized. If, however, large quantities of carbohydrate entered the circulation, two molecules of acetaldehyde might be condensed to form butyric acid in accordance with the suggestion of Magnus-Levy. The building of higher fatty acids from butyric acid would require the addition of radicles consisting of  $-\text{CH}_2-\text{CH}_2-$ . Whatever may be the character of the intermediary changes, it is evident that the removal of  $\text{CO}_2$  from methyl glyoxal would leave the radicle  $-\text{CH}_2-\text{CH}_2-$ .



The high respiratory quotient after giving carbohydrate in excess is due to this intermediary liberation of carbon dioxide.

The process of the conversion of carbohydrate into fat does not appear to be one involving any considerable increase in total heat production. Seventy grams of glucose were given to a dog on three different occasions. The extra carbon dioxide elimination which was not due to protein and carbohydrate combustion and which gave the high respiratory quotients always found after large carbohydrate ingestion, amounted to 0.8, 1.07, and 1.53 liters respectively, whereas the heat production as calculated amounted to 75.3, 75.7, and 75.6 calories. The height of the respiratory quotient is therefore not an index of increased metabolism.

If one accepts Bleibtreu's formula as the simplest expression of the conversion of carbohydrate into fat,

$$\begin{aligned} 270.6 \text{ gm. glucose} &= 100 \text{ gm. fat} + 115.45 \text{ gm. CO}_2 + 54.6 \text{ gm. H}_2\text{O} \\ 997.2 \text{ calories} &= 950.0 \text{ calories} \end{aligned}$$

the reaction is evidently exothermic, 4.7 per cent of the heat being liberated. If the heat evolved be measured on the basis of the extra carbon dioxide production, 1 liter of such carbon dioxide would have a value of 0.8 calories, or less than one-sixth the caloric equivalent of a liter of carbon dioxide obtained from the oxidation of glucose in the ordinary manner.

This discussion makes it evident that when carbohydrate is converted into fat the heat production may be calculated from the oxygen absorption with a slight addition for the exothermic production of heat based on the elimination of carbon dioxide in excess of the requirement of a non-protein respiratory quotient of unity. Such calculations show that direct and indirect calorimetry agree within 1 per cent. These experiments indicate that there is a definite upper limit for the metabolism of glucose molecules above which limit fat formation may occur from the assembled methyl glyoxal molecules which are present in excess, and that this formation of fat is accompanied by only a very slight increase in the heat production.

Benedict has stated that the cause of the specific dynamic action of carbohydrate is due to acid stimulation, partly basing his argument upon an experiment in which he gave 100 grams

of fructose to a diabetic and witnessed an increase in metabolism of 30 per cent, although the fructose was not oxidized but was completely eliminated as glucose in the urine. It may be noted that this 30 per cent increase in heat production is very much greater than that which usually occurs in the human being after food ingestion. Thus, DuBois has found that the ingestion of 200 grams of glucose by a normal man causes the metabolism to rise only 10 per cent. Furthermore, when 10 grams of fructose are given to a phlorhizinized dog there is no increase in the heat production, though the fructose is completely eliminated in the urine in the form of glucose. This contrasts with a large increase in metabolism when 12.5 grams of glycocoll, which are also convertible into 10 grams of glucose, are ingested in phlorhizin glycosuria. If it be permitted to assume that methyl glyoxal molecules are the intermediary products between fructose and glucose, then methyl glyoxal exerts no specific dynamic action in the sense of being a chemical stimulus of metabolism. The experiment performed by Benedict requires repetition and confirmation.

As regards the behavior of the different sugars in the normal organism, the work of Johansson and of Benedict has been confirmed in the demonstration that fructose induces a greater heat production than glucose. Johansson gave the probable explanation: glucose was immediately ready for glycogen formation and fructose could not be immediately so deposited and was therefore oxidized in higher measure. This idea may now be amplified if one conceives that glucose may be at once removed and stored as glycogen, whereas fructose must first be entirely converted into the more readily oxidizable methyl glyoxal molecules, affording thereby an immediate and plentiful supply of small molecular fragments available either as food for the cells or as material for the construction of glucose or glycogen.

It may be incidentally remarked that it was found that galactose was not readily oxidized by the dog, and that lactose had no effect whatever upon its metabolism, indicating the absence of lactase from the intestine.

Experiments were instituted in which the effect of the ingestion of 50 and 70 grams of glucose was compared with the effect of 50 grams of glucose to which 20 grams of glycocoll or of alanine

were added. Fifty grams of glucose increased the heat production 30 per cent, and 70 grams 35 per cent. There was little difference. Twenty grams of glycocoll increased it 36 per cent, and the same amount of alanine 32 per cent. Combined, 50 grams of glucose and 20 grams of glycocoll are the glucose equivalent of 66 grams, and yet when they were given together the metabolism increased 56 per cent. Glucose and alanine in similar quantities are a glucose equivalent of 70 grams and caused an increase of 53 per cent in the heat production. It is obvious that an increase in the quantity of glucose when this is given in large amounts, scarcely affects metabolism, whereas when the chemical stimulus from the metabolism products of the amino-acids acts on the cells in conjunction with a plentiful supply of glucose, the resultant effect is nearly equal to the sum of the two individual influences. This points to a distinct difference between the cause of the specific dynamic action of glucose and that of alanine, which latter is convertible into lactic acid and eventually into glucose.

It will be recalled that Ward has shown in a mountaineering trip on Monte Rosa that the carbon dioxide tension in the alveoli falls as an accompaniment of the rising acid content of the body. If there were an acid production after carbohydrate ingestion one would expect to find the carbon dioxide content of the blood reduced from the normal, whereas unpublished experiments of Dr. A. L. Meyer show that this is not the case, the quantity remaining unchanged. One may accept this as added proof that the specific dynamic action of carbohydrate is not due to acid stimulation.

Lactic acid from alanine or glycollic acid from glycocoll may therefore raise the level of cell activity through direct stimulation, and if fragments of glucose metabolism be present in quantity these may enter as increased fuel to produce yet higher metabolism in the cells than the oxyacids would alone induce.

Also, when alcohol is given with glucose the metabolism rises above the level it would have attained had glucose been administered alone. The respiratory quotient falls and the cells oxidize both alcohol and the fragments of glucose metabolism, and produce almost as much extra heat as the sum of the quantities of heat which each material would have induced alone.

Of such nature is the metabolism of plethora. The influx of carbohydrate or fat or of alcohol enables the cells to oxidize at a higher level through the increased mass action of food particles which are available. On the other hand, the metabolism products of glycocoll and alanine may directly stimulate protoplasm without themselves being involved in the oxidative process and this is called amino-acid stimulation.

Finally, an analysis of the results obtained on diabetic patients by Benedict and Joslin shows that the increase in metabolism which has been reported as 15 per cent is only 5 or 10 per cent. Benedict ascribes this increase to acidosis. In the phlorhizinized dog the metabolism may increase 70 per cent, in the depancreatized animal 40 per cent above the basal metabolism. Yet depancreatized animals manifest scarcely any acidosis while the tissues of the human diabetic may be filled with the acetone bodies, as has been shown by Marriott. The increase in metabolism in dogs is better explained as being due to the increased protein metabolism, as was first suggested by Rubner, and to the increased fat content of the blood; that is to say, to the dual mechanism of amino-acid stimulation and an exsistant plethora of food particles.

## ABSTRACT OF SCIENTIFIC PROCEEDINGS.

### THE EXCRETION OF CREATINE DURING A FAST.

By F. D. ZEMAN AND PAUL E. HOWE.

*(From the Biochemical Laboratories of Columbia University at Teachers College and the College of Physicians and Surgeons, New York.)*

Recent criticism<sup>1</sup> of the results obtained with the Folin method for the determination of creatine in urine in the presence of acetone and aceto-acetic acid has thrown doubt upon the presence of creatine in the urine of fasting man. We have determined creatine in the urine of a fasting man throughout a seven day fast. The method of Graham and Poulton was employed for the removal of acetone and aceto-acetic acid and quantitative determinations were made of these substances together, and of  $\beta$ -hydroxy butyric acid. Control experiments were made with untreated urine. Determinations before and after the appearance of the interfering substances showed the method to be accurate in their absence. Creatine was excreted on each fasting day in amounts comparable in most cases with those obtained in previous fasts under similar conditions.

### DETERMINATION OF CREATININE AND CREATINE; THE OCCURRENCE OF CREATINE.

By J. LUCIEN MORRIS.

*(From the Laboratory of Biological Chemistry, Washington University, St. Louis.)*

Precipitation of creatinine as creatinine potassium picrate was made the basis of a method for determining creatinine and creatine. Such an estimation of creatine demonstrated the actual presence of this substance which is hydrolyzed into creatinine, and precludes the possibility of confusion with other sub-

<sup>1</sup> G. Graham and E. P. Poulton: *Proc. Roy. Soc., series B*, lxxxvii, p. 205, 1914.

stances which are converted into forms giving the color reaction with picric acid and sodium hydroxide. The method served to separate the creatinine (excepting a practically constant fraction) from the interference of acetone bodies, and total creatinine from interference of frequently occurring undesirable products of hydrolyzing action (especially those arising from glucose). Thus separated, the double creatinine salt was brought into solution and the creatinine value determined as in the modified Folin method. Freedom from such interfering influences as these makes the method unquestionably worth while when their presence is suspected.

Satisfactory application of the method was made in the analyses of normal urines (plus creatine) with large amounts of sodium aceto-acetate and glucose added, diabetic urines, post-partum urines, and children's urines. In none of these did any interfering substance cause increase or decrease of the apparent values of either creatinine or creatine. In all classes of urines here mentioned the occurrence of creatine was demonstrated.

#### THE INFLUENCE OF PROTEIN FEEDING ON THE ELIMINATION OF CREATINE IN STARVATION.<sup>2</sup>

By WILLIAM C. ROSE.

*(From the Laboratories of Biological Chemistry of the University of Pennsylvania, Philadelphia, and of the University of Texas, Galveston.)*

Experiments on dogs indicate that the feeding of diets rich in protein after complete extirpation of the pancreas does not cause the disappearance of urinary creatine.

Contrary to the observations of Cathcart, it has been found that in man the feeding of large amounts of protein causes a marked diminution in the creatine elimination during starvation. In severe cases of diabetes, however, protein feeding in man has no influence on the output of creatine.

These results agree with those of Benedict and Osterberg on dogs with phlorhizin diabetes, and demonstrate that the disappearance of creatine from the urine following protein feeding in the normal fasting dog and in man is due to the carbohydrate arising from protein in metabolism.

<sup>2</sup> Read by title.



**THE NEPHELOMETRIC ESTIMATION OF PURINE BASES, INCLUDING URIC ACID, IN BLOOD AND URINE.<sup>1</sup>**

BY SARA STOWELL GRAVES AND PHILIP ADOLPH KOBER.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

I. Salskowski's reagent has been modified to meet nephelometric conditions, and it is shown that the modified reagent will precipitate xanthine, hypoxanthine, guanine, adenine, and uric acid in very dilute solutions (0.002 per cent) quantitatively.

II. The use of a protective colloid has been introduced—clear solutions of egg albumin—for the purpose of keeping precipitates in suspension so that they may be estimated nephelometrically.

III. It is shown that an alkaline suspension of manganese dioxide, instead of an acid medium as has been used heretofore, will oxidize uric acid completely in three minutes, leaving the other purines unattacked.

IV. It is shown that manganese dioxide is an excellent reagent for the removal of alkaline sulphides from solution without the usual boiling technique.

V. It is shown that uric acid and other purine bases in urine may be quickly and fairly accurately estimated with the nephelometer.

VI. It is shown that five volumes of 3 per cent sulphosalicylic acid is an excellent reagent for removing all coagulable protein from blood. By centrifuging for one or two minutes after the precipitation with sulphosalicylic acid, the great bulk of protein can be removed, and if the supernatant liquid is shaken with a little talcum powder to cause agglutination of any remaining suspended protein, a perfectly clear filtrate can be obtained in five to ten minutes without boiling.

<sup>1</sup> Read by title.

**DEXTROSE CONTENT OF THE EGG OF THE COMMON FOWL.<sup>4</sup>**

By M. E. PENNINGTON, N. HENDRICKSON, E. L. CONNOLLY,  
AND B. M. HENDRIX.

*(From the Food Research Laboratory, Bureau of Chemistry, U. S. Department of Agriculture, Philadelphia.)*

The dextrose of the whites of fresh hens' eggs is found to vary from 0.3 to 0.6 of 1 per cent, averaging 0.44. The yolk contains from 0.08 to 0.24, averaging 0.08. During incubation the amount decreases in fertile, but is unaffected in non-fertile eggs. Commercial frozen egg contains from 0.28 to 0.38 of 1 per cent, averaging 0.34. The eggs rejected as unfit for food, except musty eggs, contain much less than the good eggs. Storage of shell eggs for periods up to six months at a temperature close to 32° does not affect the dextrose content unless the eggs are infected by microorganisms. In commercial dried egg the amount of dextrose present does not indicate the quality of the eggs from which the product was prepared, because a considerable part of the dextrose may be lost in the process of drying. In commercial frozen egg allowed to spoil, the dextrose disappears as decomposition progresses. Yolks decompose more readily than whites. A cleared solution in which dextrose can be determined is prepared by coagulating with heat and a little acetic acid, adding a large amount of washed alumina cream, and filtering.

**A METHOD FOR DETERMINING AND COMPARING THE LOCAL TOXICITY OF CHEMICAL COMPOUNDS.**

By H. J. CORPER.

*(From the Otho S. A. Sprague Memorial Institute and Pathological Laboratory of the University of Chicago.)*

During the course of investigations on the pharmacological action of various copper amino-acid compounds it was found desirable to study the local toxicity of these compounds and to compare their action with that of copper salts of simple composition; *i.e.*, copper sulphate. The method used in intracutaneous tuberculin tests was admirably adapted for this purpose, requiring a 1 cc. tuberculin syringe graduated in 0.01 cc. and

<sup>4</sup> Read by title.

fitted with a platinum needle (preferred for its resistance to the action of chemicals). Any suitable animal (preferably white and with the back shaved) is given intracutaneous injections of 0.2 cc. of varying concentrations of the two compounds to be tested and compared (using, for instance, 1.0, 0.1, 0.01, and 0.001 per cent salt concentration), one compound being injected on one side of the back and the other on the opposite side, equal concentrations opposite each other, and noting from day to day the extent and type of the tissue changes produced.

Thus it was noted that the local toxicity of a copper amino-acid mixture prepared from hydrolyzed egg albumin did not greatly differ from that of copper sulphate, the former producing, however, a hemorrhagic lesion while the latter produced a simple necrosis.

H. L. Huber continuing this investigation has found the same to be true of the leucinate and glutamate of copper.

#### DIGESTIVE PROCESSES IN LIMULUS.<sup>5</sup>

BY HELEN I. MATTILL AND H. A. MATTILL.

*(From the Marine Biological Laboratory, Woods Hole, and the Laboratory of Physiological Chemistry, University of Utah, Salt Lake City.)*

The alimentary tract comprises esophagus, stomach, intestine and end-gut; all are chitinous except the long straight intestine. A pyloric valve is invaginated into the intestine. From the intestine two pairs of ducts lead into the large digestive gland ("liver") which is inextricably intertwined with the gonads; this mass fills the body cavity. The entire tract is alkaline. By means of a glass tube a red brown liquid is sometimes obtainable from the stomach, which possesses the same digestive powers as the intestinal contents, a protease (leucine and tyrosine from fibrin), an amylase-maltase (dextrose from starch), and a lipase (litmus-milk). Extracts of the intestinal mucosa and of the digestive gland showed the same digestive activity. The source of the secretion and the place of absorption are not yet certain. The intestine is filled with a clear, formless, jelly-like mass which completely invests and infiltrates the intestinal contents, and

<sup>5</sup> Read by title.

which has perhaps the same function as the peritrophic membrane in insects, protecting the epithelium and allowing dissolved material to pass out, and into the digestive gland. This jelly does not show carbohydrate on hydrolysis. Further work is in progress and histological material is in preparation.

#### EXPERIMENTS WITH dl-GLYCERIC ALDEHYDE.

By R. T. WOODYATT.

*(From the Otho S. A. Sprague Memorial Institute Laboratory of Clinical Research, Rush Medical College, Chicago.)*

Pure crystalline *dl*-glyceric aldehyde made by a modified Wohl synthesis by E. J. Witzemann is capable of complete conversion into *d*-glucose in the fully diabetic organism, if given in low dilutions over a long time. If given in high concentration it fails to appear as glucose and apparently burns. By mouth doses of 1.7 gm. per kilo of body weight caused diarrhea with unchanged triose in the stool and suppression of urine. Doses of 2.5 gm. per kilo subcutaneously are lethal, but cause no diarrhea and no excretion of unchanged triose by any route.

#### THE LEVEL OF THE SUGAR IN THE BLOOD FLOWING FROM THE LIVER UNDER LABORATORY CONDITIONS.

By J. J. R. MACLEOD AND R. G. PEARCE.

*(From the Physiological Department, Western Reserve University, Cleveland.)*

In order that the mobilization of sugar from the liver may be more thoroughly studied than has previously been the case, it is necessary to estimate the reducing power in small quantities of blood taken at short intervals from the inferior vena cava opposite the hepatic veins. By a modification of Bang's micro method we have been able to do this with satisfactory accuracy in samples of blood measuring 2 cc. each and removed every five minutes. The results obtained so far are as follows:

1. The experimental error of the method does not usually amount to more than 5 per cent. Occasionally it has been found to be greater. It is determined frequently for each experiment.

2. In a period of at least thirty minutes following the etherization and operative preparation of the animals, there was found to be a progressive fall in the sugar concentration. In observations on six dogs, this fall, expressed as a percentage of the original amount of sugar, amounted to 13, 14, 7.5, 10.5, and 23. The operations referred to consisted in introducing tracheal, carotid, and vena cava cannulae.

3. After the first half hour, in four of the experiments there was a more or less distinct rise in sugar concentration, which was sufficient to bring it back to the original level in about one and one-half hours in three of the experiments. In the remaining two experiments the original decline persisted.

4. It has been impossible to correlate these changes with any observable alteration in the physiological condition of the animals (arterial blood pressure, rectal temperature, respirations, depth of anesthesia).

5. The extent of percentile rise occurring during a period of 10 minutes varied as follows: 5.6, 4.5, 9.8, 6.5, 7, 11.5. These values are considerably below those previously obtained by us in observations made on blood similarly removed from animals in which the splanchnic or hepatic nerves were stimulated.

**A METHOD FOR THE DECOMPOSITION OF THE PROTEINS  
OF THE THYROID WITH A DESCRIPTION OF CERTAIN  
CONSTITUENTS.**

By E. C. KENDALL.

*(From the Mayo Clinic, Rochester, Minn.)*

The proteins of the thyroid may be decomposed into simpler products by hydrolysis with 1 per cent sodium hydroxide in the presence of 90 per cent ethyl alcohol. The products so obtained may be separated into two groups by their solubility in acids. Those compounds precipitated by acids are designated Group A; those compounds soluble in acids are designated Group B.

The iodine in the thyroid is evenly divided between these two groups, but there are many differences in the chemical properties of A and B iodine. Group A constituents are essentially acidic. Purification removes tryptophane, tyrosine, and lauric acid. The percentage of iodine is thus increased, and from these puri-

fied products I have isolated a crystalline compound containing 60 per cent of iodine. Group B consists of amino-acid complexes, most of which are precipitated with 40 per cent ammonium sulphate, but they are easily dialyzable. There is also a compound which reduces silver and mercury salts. I have named this compound R.

The physiological activity of the thyroid is still retained in the constituents of A and B. In myxedema the dry, scaly skin is restored to moist normal condition by B. R, one of the constituents of B, relieves muscle cramp. No toxic symptoms can be produced by any of the constituents of B.

Marked toxic symptoms can be produced by A; namely, greatly increased pulse rate, loss of weight, nervousness, nausea, diarrhea, and severe headaches. The severity of these symptoms depends upon the iodine content of A.

#### THE RELATION OF HYDROCHLORIC ACID TO THE MORPHOLOGICAL CHANGES INDUCED BY CHLOROFORM.

By EVARTS A. GRAHAM.

(From the Otho S. A. Sprague Memorial Institute Laboratory of Clinical Research, Rush Medical College, Chicago.)

Central lobular liver necrosis of the type found in "late chloroform poisoning" has been produced experimentally by other aliphatic halogen substituted compounds; *viz.*,  $\text{CH}_2\text{Cl}_2$ ,  $\text{CCl}_4$ ,  $\text{CHBr}_3$ ,  $\text{CHI}_3$ ,  $\text{C}_2\text{H}_5\text{Cl}$ ,  $\text{C}_2\text{H}_5\text{Br}$ ,  $\text{C}_2\text{H}_5\text{I}$ ,  $\text{C}_2\text{H}_4\text{Br}_2$ . It could not be produced with ether or chloral hydrate. Chloroform *in vitro* is decomposed by oxygen into  $\text{HCl}$  and  $\text{COCl}_2$ . The latter is further changed by water into  $2\text{HCl}$  and  $\text{CO}_2$ . Analogous decompositions occur with the other drugs named. The necrosis, and perhaps also the edema, hemorrhages, and fat infiltration which accompany it, are held to depend mainly on the action of the respective halogen acids formed in the breakdown of these substances in the body.

1. Oral or intraportal administration of  $\text{HCl}$ , in suitable concentrations, produces liver necrosis, edema, hemorrhages, and fat accumulation.

2. In chloroform poisoning central necrotic areas in the liver show a higher  $\text{H}^+$  and  $\text{Cl}^-$  content than control normal livers

when the  $H^+$  content is determined by neutral red and naphthol blue, and the  $Cl^-$  content by  $AgNO_3$ .

3. The formation of the respective halogen acid after the administration of each of the drugs above mentioned is shown by the occurrence of the neutral salts of these acids in the urine.

4. Alkali given in proper concentration inhibits the production of the necrosis and other changes by chloroform.

5. The powers of  $CCl_4$ ,  $CHCl_3$ , and  $CH_2Cl_2$  to produce these changes are proportional to the amounts of  $HCl$  which they may yield by decomposition; *viz.*, in the order mentioned.

6. Of the ethyl compounds the chloride is least powerful in this respect and the iodide the most powerful. This agrees with their respective ability to form  $HCl$ ,  $HBr$ , and  $HI$  outside of the body.

7. The failure to produce the same type of changes with chloral hydrate agrees with its failure to form an appreciable amount of  $HCl$  in its breakdown in the body.

**VARIATIONS IN FACTORS ASSOCIATED WITH ACIDITY OF  
HUMAN URINE DURING A SEVEN DAY FAST AND  
DURING THE SUBSEQUENT NON-PROTEIN AND  
NORMAL FEEDING PERIODS.**

BY F. D. ZEMAN, JEROME KOHN, AND PAUL E. HOWE.

(From the Biochemical Laboratories of Columbia University at Teachers College and the College of Physicians and Surgeons, New York.)

A study was made of the variations in acidity (true and titratable) of human urine with relation to the modifying factors present during fasting and recuperation. The range of variations of the acidity extended from a fairly acid urine,  $p_H$  5.1 (third day of fast) to an alkaline urine,  $p_H$  8.0 (last day of the final period). The diet of the preliminary and final feeding periods was the same in nature, as that used in previous experiments.<sup>6</sup> In the non-protein period cane-sugar, clarified butter, salts (alkaline mixture), and agar-agar were ingested.

Determinations were made of the  $H^+$  ion concentration (indi-

<sup>6</sup> P. E. Howe, H. A. Mattill, and P. B. Hawk: *Jour. Am. Chem. Soc.*, xxxiii, p. 568, 1911. Howe and Hawk: *Proc. Am. Soc. Biol. Chemists*, 2, p. 65, 192, this *Journal*, xi, p. xxxi, 1912.

cators); titratable acidity or alkalinity, using (a) phenolphthalein, (b) neutral red, (c) methyl orange; phosphates, ammonia, acetone, aceto-acetic acid, and  $\beta$ -hydroxy butyric acid.

In the absence of exogenous phosphorus (fasting) we found the acidity (true and titratable), phosphates, acetone, aceto-acetic acid, and total nitrogen to vary together. During the non-protein post-fasting period we found an increased  $H^+$  ion concentration and acidity without an accompanying increase in the nitrogen excretion; acetone and aceto-acetic acid were absent. The increased excretion of ammonia in fasting is correlated with that of  $\beta$ -hydroxy butyric acid; when not influenced by this factor, as in the preliminary, non-protein, and final feeding periods, the ammonia excretion fluctuated with the  $H^+$  ion concentration and the acidity. The low ammonia excretion in the final period showed the low  $H^+$  ion concentration and titratable acidity to result from a loss of fixed base. This phenomenon is apparently characteristic of recuperation (nitrogen retention).

It seems probable that the increased nitrogen excretion during the early days of a fast of a human individual is related to the metabolic processes which result in the excretion of aceto-acetic acid.

#### ON THE INFLUENCE OF SODIUM CARBONATE UPON GLYCO-SURIA, HYPERGLYCEMIA, AND THE RESPIRATORY METABOLISM OF DEPANCREATIZED DOGS.

BY B. KRAMER AND J. R. MURLIN.

(From the Physiological Laboratories of Cornell University Medical College, New York City, and of the University of Iowa, Iowa City.)

The present paper is a brief report of a large number of experiments on the influence of sodium carbonate administered intravenously, subcutaneously, or orally on the fate of glucose in the depancreatized dog. The initial observation that 150 cc. of a 1 per cent solution of sodium carbonate greatly reduces the excretion of glucose in such an animal was reported a year and a half ago. To determine the fate of this retained glucose we naturally examined the blood first. Later, after greatly reducing the sugar in the urine, either by large doses given orally or by



smaller doses given intravenously, the respiratory exchange was studied before and after the administration of pure glucose by stomach. Finally, the liver and muscles have been examined for glycogen, the feces have been examined for sugar, and the urine has been tested for lactic acid.

#### CONCLUSIONS.

1. The administration of sodium carbonate to the depancrea-tized dog lowers the D:N ratio as well as the sugar output per hour.

2. That this is not due to a decrease in the permeability of the kidneys is shown by the fact that there is no increase and often a fall both in the percentage and absolute quantity of sugar in the blood.

3. After the administration of carbonate and sugar a definite retention of glucose occurs which is not due to its conversion into glycogen unless it be retained as glycogen in organs other than the liver or muscles.

4. The sugar which thus disappears from the urine is not excreted into the alimentary tract nor does it undergo condensation into substances that can be hydrolyzed by dilute acids.

5. In the urines examined no lactic acid could be found even when the D:N ratio was minimal.

6. For the time that the respiratory gases were studied the quotients obtained would indicate the combustion of but a minimal amount of glucose, if any.

That glucose may be burned in the diabetic dog after the repeated administration of sodium carbonate is a probability, or that it may be converted into intermediary compounds other than lactic acid is another possibility, and these will furnish the subjects for future investigation.

**NARCOTICS IN PHLORHIZIN DIABETES.**

BY W. D. SANSUM AND R. T. WOODYATT.

*(From the Otho S. A. Sprague Memorial Institute Laboratory of Clinical Research, Rush Medical College, Chicago.)*

The administration of phlorhizin alone in fasting does not remove all glycogen from the body. This can be accomplished by subcutaneous injections of epinephrin (0.25 mgm.) at three hour intervals until the epinephrin no longer affects glycosuria. Any drug which in non-diabetic animals can cause hyperglycemia can also cause completely phlorhizinized, but not deglycogenized dogs to excrete "extra sugar."

Ether, nitrous oxide, acetaldehyde, etc., (narcotics) do this by virtue of their power to produce tissue asphyxia (increased acid). Among substances now regarded as capable of conversion into glucose a number have been tested only in incompletely phlorhizinized dogs, and react like ether, etc. Pyruvic acid and acetaldehyde are not sugar formers.

**ACCELERATION OF LIVER AUTOLYSIS.**

BY H. C. BRADLEY.

*(From the Department of Physiology, University of Wisconsin, Madison.)*

Normal liver autolyzes till about 25 per cent of the nitrogen is not precipitated by tannic acid. The reaction is practically at equilibrium before ten days, and changes but slightly after three days. The addition of acid, or manganese salts leads to a more rapid and more complete hydrolysis. This increase is roughly proportional to the amount of acid or salt added until an optimum concentration is reached. In the case of HCl this optimum is about 1/50 mol.; in the case of  $MnCl_2$  it is about 1/10 mol. At these concentrations the two reactions are nearly identical. A similar increase of soluble nitrogen results from the addition of peptone, casein, and boiled liver. The addition of ovalbumin or edestin leads to no increase in soluble nitrogen. The former proteins constitute additions to the available substrate; the latter are not hydrolyzed at all by the enzymes and are not available in the reaction. The addition of the former gives

an accelerated reaction which attains a higher soluble nitrogen level, and thus resembles the reaction in the presence of acid or  $\text{MnCl}_2$ .

The evidence appears to indicate that in the normal liver there are three protein groups or fractions. One represents the connective tissue; another a mixture of hemoglobin and albumins; and the third a globulin type of protein. The connective tissue is insoluble and not attacked by the proteases under any of the conditions tried. It is, therefore, in no sense a substrate. It contains about 10 to 25 per cent of the total nitrogen of the liver. The second fraction, 45 to 65 per cent of the nitrogen, is not digested under normal conditions. It also is not to be considered substrate and has no mass effect in determining speed or equilibrium of the reaction. It may, however, be rendered available by acids, salts of manganese, and other electrolytes, and when available acts as an added mass of substrate leading to more rapid digestion and a larger amount of soluble nitrogen. The third fraction, comprising from 25 to 30 per cent of the total nitrogen, is readily available and normally is completely hydrolyzed under the experimental conditions in a relatively short time,—about two weeks. It is the mass of this fraction which determines the speed and equilibrium of normal liver autolysis.

It is believed that most of the accelerations reported in the literature involve a shift upward of the equilibrium as well, and are only intelligible on the assumption that some of the non-available proteins are converted into substrate. In such conditions as phosphorus poisoning and long chloroform narcosis, the marked liver degenerations are due, first, to the toxic action on liver cells resulting in their death, and second, to the production of acid sufficient to neutralize the alkalinity of the cells—which inhibits autolysis—and to convert in addition some of the non-available proteins into available. The result is a rapid and extensive necrosis *in vivo*, while such tissue *in vitro* autolyzes faster and more completely than normal liver.

There seems to be no evidence of an activation of a zymogen in these cases.

ON THE NATURE OF THE HEPATIC FATTY INFILTRATION IN  
LATE PREGNANCY AND EARLY LACTATION.

By V. H. MOTTRAM.

During pregnancy sections of liver show an increase of fat under the microscope (Miotti). This increase is often very striking, occurs also in lactation; and simulates hunger and post-chloroform poisoning infiltrations. Cats and rabbits from the ordinary sources demonstrate this clearly.

In an early experiment no less than 24 per cent of the fresh liver tissue was found to be pure non-volatile fatty acid with low iodine value. As the work developed the following data were collected: period of gestation; body and liver weights, percentage of pure non-volatile fatty acids of the liver; iodine values of mesenteric hepatic and kidney fatty acids.

Cats show too wide a variation from the mean in normal animals to yield unquestionable evidence in favor of infiltration. They require too much time and accommodation to bring their metabolism to an undisturbed level upon which a possible infiltration might write itself. Rabbits are easier material. A month of quietude, separation, and ample dietary is enough to reduce individual variation within bounds.

Control animals have a fatty acid percentage of fresh liver ranging from 2.36 to 3.19, with a mean of 2.62. Animals treated in the same way for the same time or longer, at full term or a day or two post-partum, show a range from 2.67 to 6.05, with a mean of 4.43. Taking other criteria, *e.g.*, total liver fat or total liver fat divided by a function of the body weight, the result is the same: *at or about the time of parturition there is an increase in the total fat in the liver.* Histological results parallel this even in small percentage deviations. Argument from iodine values shows that the liver is infiltrated with fatty acid.

**THE SYNTHESIS OF HIPPURIC ACID IN EXPERIMENTAL TARTRATE NEPHRITIS IN THE RABBIT.**

By F. B. KINGSBURY AND E. T. BELL.

*(From the Departments of Physiology and Pathology of the Medical School of the University of Minnesota, Minneapolis.)*

Male rabbits, kept on a diet of carrots, were injected subcutaneously daily with sodium benzoate for a fore-period of several days, and then injected with from 0.3 to 0.7 of a gram of racemic tartaric acid dissolved in water and neutralized with sodium carbonate. This was injected into the muscles of the back. On the day that the tartrate was injected, the injection of sodium benzoate was postponed for two or three hours, for it was found that if it were injected at the same time as the tartrate, it prevented the production of a severe nephritis.

The urines were analyzed during the fore-period and during the period of nephritis, usually two or three days, for total nitrogen, total benzoic, free benzoic, and hippuric acids. The rabbit was then killed and an autopsy of its kidneys made.

The degree of nephritis was shown by the phenolsulphone-phthalein test of Rowntree and Geraghty. A trace or zero of phthalein excretion is always associated with severe injury to the convoluted tubules, as shown by Potter and Bell.

Severe nephritis of the convoluted tubules, as indicated by a zero or trace of phthalein excretion and confirmed by autopsy, did not affect materially the synthesis and excretion of hippuric acid. This is in agreement with the findings of Saloman and of Jaarsveld and Stokvis, that in the rabbit hippuric acid is synthesized in other places as well as in the kidneys.

**ON THE RELATION OF THE OXYGEN TENSION OF THE ATMOSPHERE TO COMBUSTION.**

By H. C. DALLWIG, A. C. KOLLS, AND A. S. LOEVENHART.

*(From the Pharmacological Laboratory of the University of Wisconsin, Madison.)*

Since the time of Lavoisier much work has been done to show the similarities and differences between oxidation within and without the organism. In connection with our work on the effect of decreased oxygen tension in the respired air on the erythro-

cytes and hemoglobin, we exposed animals for one to two weeks to atmospheres of 10 per cent oxygen and 90 per cent nitrogen at the atmospheric pressure. We also exposed animals to atmospheres of similar low oxygen tension by partially evacuating the respiratory chamber. We found that the physiological response is identical whether correspondingly low oxygen pressures are obtained by diluting the air with nitrogen or by partially evacuating the chamber. We determined to study also the effect of low oxygen tensions on the candle flame and the flames of other common combustible material. It was shown by Clowes,<sup>7</sup> working at atmospheric pressure, that various combustible gases and liquids would cease to burn at definite minimal concentrations of oxygen. We have confirmed this. We have found that the paraffin as well as the ordinary candle is extinguished within a few seconds in an atmosphere of 15.5 per cent O<sub>2</sub> and 84.5 per cent N<sub>2</sub>; viz., at an oxygen tension of 116.4 mm. Hg. We have found that by rapidly evacuating a vessel filled with air the flame is extinguished at a pressure of the residual air of 95 mm. Hg., which corresponds to an oxygen tension of 19.8 mm. Hg., or 2.6 per cent of an atmosphere.

#### CONCLUSIONS.

1. In certain combustions at least nitrogen is a powerful inhibiting factor entirely apart from the proposition that it dilutes the oxygen. Thus the flame of the candle is just extinguished at 116.4 mm. Hg. oxygen pressure when the oxygen pressure is lowered by the addition of nitrogen, whereas the flame is just extinguished at 19.8 mm. Hg. oxygen pressure if the lowering is effected by evacuation. In other words, the excess nitrogen retards the combustion enormously and in the presence of this gas in amounts required to make up the atmospheric pressure a partial pressure of oxygen six times as great as in the evacuated atmosphere has the same value in supporting combustion.

2. The results bring out a striking difference between combustion and vital oxidation inasmuch as the latter depends purely on the oxygen tension, whereas in the former the nitrogen tension plays an important rôle.

<sup>7</sup> Clowes and Redwood: *Detection and Estimation of Inflammable Gas and Vapor in the Air*, London, 1896.

## THE LEVEL OF BLOOD SUGAR IN THE DOG.

BY P. A. SHAFFER AND R. S. HUBBARD.

(From the Laboratory of Biological Chemistry, Washington University,  
St. Louis.)

Results already reported<sup>8</sup> show that the *normal* level of blood sugar of the dog is about 0.05 per cent, but that if the animals are anesthetized with ether and the blood is drawn after an incision and dissection of a vessel, the amount of sugar found is usually between 0.10 per cent and 0.20 per cent, the values usually, but erroneously, accepted as normal. Later experiments appear to indicate that the higher results are in large part due to mild degrees of asphyxia, even in those cases when the animal seems to be breathing well. If a tracheal cannula is inserted at once after the animal is anesthetized, and forced respiration is maintained, the blood sugar may rise slightly, but does not reach the values found without the forced respiration.

The value of these observations would appear to be that by forced respiration the normal level of blood sugar of the dog may be almost, though not quite, maintained during operative procedures lasting even for several hours.

It is suggested that forced respiration should be established in all experiments on dogs in which variations of the level of blood sugar are being observed as the result of any operative procedure.

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<sup>8</sup> P. A. Shaffer: this *Journal*, xix, p. 297, 1914.

<sup>9</sup> Presented from the Physiological Society in joint session of the Federation.

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<sup>11</sup> Presented from the Pharmacological Society in joint session of the Federation.

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<sup>19</sup> Presented from the Pathological Society in joint session of the Federation.

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**THE JOURNAL OF BIOLOGICAL CHEMISTRY**

**STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS  
AS DISPLAYED IN THE COMPOSITION OF  
THE SERUM PROTEINS.**

**III. A COMPARISON OF THE SERA OF THE HEN, TURKEY, DUCK,  
AND GOOSE WITH RESPECT TO THEIR CONTENT  
OF VARIOUS PROTEINS.**

BY W. B. THOMPSON.

(*From the Rudolph Spreckels Physiological Laboratory of the University of  
California.*)

(Received for publication, October 28, 1914.)

The following investigations were undertaken at the suggestion of Dr. T. Brailsford Robertson, and constitute a third paper in a series of "Studies in the Blood Relationship of Animals as Displayed in the Composition of the Serum Proteins."<sup>1,2</sup> In this work I have employed Robertson's improved refractometric method<sup>3</sup> for the quantitative determination of the various proteins contained in the sera.

In each instance the fowls were kept fasting for from eighteen to twenty hours previous to bleeding, but had free access to water. The sera were obtained by immediately defibrinating the fresh blood by shaking with glass beads. This must be done promptly, as the coagulation time for the blood of hens and ducks is about thirty seconds, for turkey blood about forty seconds, and for goose blood only about twenty seconds. Because of the small amount of blood yielded by each one of the smaller fowls, it was necessary to bleed several individuals for a sample of serum, as noted below before each table of results. The analytical results reported are, in each instance, the average of at least two closely agreeing determinations made upon the same sample of serum.

<sup>1</sup> T. B. Robertson: this *Journal*, xiii, p. 325, 1912.

<sup>2</sup> J. Homer Woolsey: this *Journal*, xiv, p. 433, 1913.

<sup>3</sup> Robertson: *loc. cit.*

## Proteins of Blood Sera

*A. Hen serum.*

The following are the results obtained from eight samples of sera from hens of between one and two years of age, each sample being from six hens.

TABLE I.  
*Hen serum.*

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.73 $\pm$ 0.04	1.12 $\pm$ 0.15	3.8 $\pm$ 0.2	5.0 $\pm$ 0.2
2	0.73 $\pm$ 0.04	1.11 $\pm$ 0.15	4.0 $\pm$ 0.2	5.2 $\pm$ 0.2
3	0.77 $\pm$ 0.04	1.18 $\pm$ 0.15	3.9 $\pm$ 0.2	5.0 $\pm$ 0.2
4	0.73 $\pm$ 0.04	1.18 $\pm$ 0.15	3.9 $\pm$ 0.2	5.0 $\pm$ 0.2
5	0.73 $\pm$ 0.04	1.12 $\pm$ 0.15	3.8 $\pm$ 0.2	5.0 $\pm$ 0.2
6	0.77 $\pm$ 0.04	1.18 $\pm$ 0.15	3.9 $\pm$ 0.2	5.0 $\pm$ 0.2
7	0.73 $\pm$ 0.04	1.12 $\pm$ 0.15	3.8 $\pm$ 0.2	5.0 $\pm$ 0.2
8	0.73 $\pm$ 0.04	1.12 $\pm$ 0.15	3.8 $\pm$ 0.2	5.0 $\pm$ 0.2
Average	0.74 $\pm$ 0.04	1.14 $\pm$ 0.15	3.9 $\pm$ 0.2	5.0 $\pm$ 0.2

In each column the percentages show the per cent of that protein for each sample of serum. The figure following the  $\pm$  sign is, in each instance, the possible error due to a possible error of one minute in reading the angle of total reflection.

Expressing each of the above proteins in terms of the percentage of the total protein content of the serum, the following figures are obtained:

	<i>per cent</i>	
"Insoluble" globulin.....14.9	$\left\{ \begin{array}{l} 15.3 \\ 14.3 \end{array} \right\}$	( $\pm 0.4$ )
Total globulins.....23.0	$\left\{ \begin{array}{l} 23.0 \\ 22.0 \end{array} \right\}$	( $\pm 2.0$ )
Total albumins.....77.0	$\left\{ \begin{array}{l} 78.0 \\ 77.0 \end{array} \right\}$	( $\pm 2.0$ )

The first figure following each protein group represents the average percentage; the upper figure immediately following is the highest percentage observed in any sample of the sera; the lower figure, the lowest percentage observed in any sample of the sera; and the figure in parenthesis, the plus or minus error in the estimate of these percentages that would be brought about by a possible error of one minute in reading the total angle of reflection.

W. D. Halliburton<sup>4</sup> estimates the proteins of hen serum to be as follows:

	<i>per cent</i>
Total globulins.....	2.90
Total albumins.....	1.21
Total proteins.....	4.14

That these figures differ in so great a degree from my results may be accounted for by the fact that Halliburton employed Hammarsten's method of estimating the total globulins. Moreover, the birds employed by Halliburton were probably not fasted.<sup>5</sup>

*B. Rooster serum.*

The following are the results obtained from determinations made upon four samples of serum from roosters aged four years and over, each sample being from three roosters.

TABLE II.  
*Rooster serum.*

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
9	0.70 $\pm$ 0.04	1.12 $\pm$ 0.15	4.9 $\pm$ 0.2	6.0 $\pm$ 0.2
10	0.63 $\pm$ 0.04	1.14 $\pm$ 0.15	5.1 $\pm$ 0.2	6.2 $\pm$ 0.2
11	0.70 $\pm$ 0.04	1.13 $\pm$ 0.15	4.7 $\pm$ 0.2	5.8 $\pm$ 0.2
12	0.70 $\pm$ 0.04	1.12 $\pm$ 0.15	4.9 $\pm$ 0.2	6.0 $\pm$ 0.2
Average	0.69 $\pm$ 0.04	1.127 $\pm$ 0.15	4.9 $\pm$ 0.2	6.0 $\pm$ 0.2

Expressing each of the above proteins in terms of the percentage of the total protein content of the serum, the following relationship is shown:

	<i>per cent</i>	
"Insoluble" globulin.....11.4	$\left\{ \begin{array}{l} 12.0 \\ 10.7 \end{array} \right\}$	( $\pm 0.4$ )
Total globulins.....19.0	$\left\{ \begin{array}{l} 20.0 \\ 18.0 \end{array} \right\}$	( $\pm 2.0$ )
Total albumins.....81.0	$\left\{ \begin{array}{l} 82.0 \\ 81.0 \end{array} \right\}$	( $\pm 2.0$ )

<sup>4</sup> W. D. Halliburton: *Jour. Physiol.*, vii, p. 319, 1886.

<sup>5</sup> Compare the succeeding article, p. 7.



This table shows a marked difference in the total protein content from that of the hen serum, the difference being chiefly in the amount of albumins present. It has been reported<sup>6</sup> for human serum that the organic content, of which over 90 per cent is protein, is greater in the male than in the female. Unfortunately it was not possible to make determinations upon the sera of fowls of the same age of opposite sexes, and upon fowls of the same sex with as great a difference in ages as exists between the ages of the hens and roosters used. Hence I am unable to state whether this difference in the proteins may be attributed to the influence of age or sex.

*C. Turkey serum.*

The following results were obtained from determinations made upon separate samples of sera from three turkeys.

TABLE III.  
*Turkey serum.*

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
13	0.47 $\pm$ 0.04	1.02 $\pm$ 0.15	5.2 $\pm$ 0.2	6.3 $\pm$ 0.2
14	0.50 $\pm$ 0.04	0.98 $\pm$ 0.15	5.4 $\pm$ 0.2	6.4 $\pm$ 0.2
15	0.47 $\pm$ 0.04	1.00 $\pm$ 0.15	5.0 $\pm$ 0.2	6.0 $\pm$ 0.2
Average	0.48 $\pm$ 0.04	1.00 $\pm$ 0.15	5.23 $\pm$ 0.2	6.2 $\pm$ 0.2

Expressing each of the above in terms of the percentage of the total protein content of the serum, the following figures are obtained:

		<i>per cent</i>	
"Insoluble" globulin.....	7.4	$\begin{Bmatrix} 7.8 \\ 6.8 \end{Bmatrix}$	( $\pm 0.4$ )
Total globulins.....	16.0	$\begin{Bmatrix} 17.0 \\ 15.0 \end{Bmatrix}$	( $\pm 2.0$ )
Total albumins.....	84.0	$\begin{Bmatrix} 85.0 \\ 84.0 \end{Bmatrix}$	( $\pm 2.0$ )

*D. Duck serum.*

The following results were obtained from determinations made upon duck sera. The number of ducks that were bled to obtain

<sup>6</sup> O. Hammarsten: *A Text Book of Physiological Chemistry*, translated by Mandel, 6th edition, New York, 1911, p. 315.

each sample of serum is noted in parenthesis after the experiment number.

TABLE IV.  
*Duck serum.*

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
16 (1)	1.41 $\pm$ 0.04	1.90 $\pm$ 0.15	4.6 $\pm$ 0.2	6.5 $\pm$ 0.2
17 (1)	1.56 $\pm$ 0.04	2.01 $\pm$ 0.15	4.6 $\pm$ 0.2	6.6 $\pm$ 0.2
18 (2)	1.53 $\pm$ 0.04	2.00 $\pm$ 0.15	5.0 $\pm$ 0.2	7.0 $\pm$ 0.2
19 (2)	1.28 $\pm$ 0.04	1.72 $\pm$ 0.15	5.0 $\pm$ 0.2	6.6 $\pm$ 0.2
20 (3)	1.44 $\pm$ 0.04	1.91 $\pm$ 0.15	4.8 $\pm$ 0.2	6.7 $\pm$ 0.2
Average	1.44 $\pm$ 0.04	1.91 $\pm$ 0.15	4.8 $\pm$ 0.2	6.7 $\pm$ 0.2

Expressing each of the above proteins in terms of the total protein content of the serum, the following figures are obtained:

		<i>per cent</i>	
"Insoluble" globulin.....	21.6	$\begin{Bmatrix} 23.8 \\ 19.1 \end{Bmatrix}$	( $\pm 0.4$ )
Total globulins.....	26.0	$\begin{Bmatrix} 31.0 \\ 26.0 \end{Bmatrix}$	( $\pm 2.0$ )
Total albumins.....	74.0	$\begin{Bmatrix} 74.0 \\ 73.0 \end{Bmatrix}$	( $\pm 2.0$ )

*E. Goose serum.*

The following results were obtained from determinations made upon separate samples from four geese.

TABLE V.  
*Goose serum.*

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
21	0.73 $\pm$ 0.04	1.11 $\pm$ 0.15	3.0 $\pm$ 0.2	4.2 $\pm$ 0.2
22	0.70 $\pm$ 0.04	1.11 $\pm$ 0.15	3.0 $\pm$ 0.2	4.2 $\pm$ 0.2
23	0.73 $\pm$ 0.04	1.08 $\pm$ 0.15	3.0 $\pm$ 0.2	4.1 $\pm$ 0.2
24	0.70 $\pm$ 0.04	1.08 $\pm$ 0.15	3.0 $\pm$ 0.2	4.1 $\pm$ 0.2
Average	0.71 $\pm$ 0.04	1.10 $\pm$ 0.15	3.0 $\pm$ 0.2	4.1 $\pm$ 0.2

Expressing each of the above proteins in terms of the total protein content of the serum, the following figures are obtained:

		<i>per cent</i>	
"Insoluble" globulin.....	17.2	$\begin{Bmatrix} 17.6 \\ 16.3 \end{Bmatrix}$	( $\pm 0.4$ )
Total globulins.....	26.0	$\begin{Bmatrix} 27.0 \\ 26.0 \end{Bmatrix}$	( $\pm 2.0$ )
Total albumins.....	74.0	$\begin{Bmatrix} 74.0 \\ 73.0 \end{Bmatrix}$	( $\pm 2.0$ )

## SUMMARY.

The following table summarizes the average results obtained.

TABLE VI.

	PERCENTAGE OF THE TOTAL PROTEINS IN THE SERA OF				
	Hen	Rooster	Turkey	Duck	Goose
"Insoluble" globulin.....	14.9	11.4	7.4	21.6	17.2
Total globulins.....	23.0	19.0	16.0	29.0	26.0
Total albumins.....	77.0	81.0	84.0	72.0	74.0

**STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS  
AS DISPLAYED IN THE COMPOSITION OF  
THE SERUM PROTEINS.**

**IV. A COMPARISON OF THE SERA OF THE PIGEON, ROOSTER,  
AND GUINEA FOWL WITH RESPECT TO THEIR CONTENT  
OF VARIOUS PROTEINS IN THE NORMAL AND  
IN THE FASTING CONDITION.**

By R. S. BRIGGS.

(*From the Rudolph Spreckels Physiological Laboratory of the University of  
California.*)

(Received for publication, October 28, 1914.)

In carrying out the following series of observations I have made use of the refractometric method devised by Robertson for the determination of the relative amount of the various blood proteins.<sup>1</sup>

For each species of animal used in this investigation, observations were taken in both the fasting and non-fasting condition, the latter being subsequently referred to as "normal." The length of the fast was in all cases at least twenty-four hours.

The serum was obtained by cutting the jugular vein, defibrinating the blood with glass beads, centrifuging, and carefully removing the serum with a bulb-pipette. In the case of the pigeon and guinea fowl it was found necessary to use more than one bird in order to obtain a sufficient amount of serum for a complete examination. For example, in the first analysis with pigeon serum the amount obtained from four pigeons was found to be insufficient, and consequently six were used for each subsequent analysis. In the case of the guinea fowls, two were used for the first and three for the second analysis. In the case of the rooster, however, a sufficient amount could be obtained from one bird for a complete analysis.

*A. Pigeon serum.*

The following numbers indicate the percentages of the various proteins present in the original serum.

<sup>1</sup> This *Journal*, xiii, p. 325, 1913.

TABLE I.  
*Normal pigeons.*

GROUP NO.	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1		$0.93 \pm 0.15$	$3.4 \pm 0.2$	$4.3 \pm 0.2$
2	$0.33 \pm 0.04$	$1.17 \pm 0.15$	$2.6 \pm 0.2$	$3.8 \pm 0.2$
3	$0.30 \pm 0.04$	$1.58 \pm 0.15$	$3.5 \pm 0.2$	$5.1 \pm 0.2$
Average	$0.32 \pm 0.04$	$1.22 \pm 0.15$	$3.1 \pm 0.2$	$4.4 \pm 0.2$

The figure following the  $\pm$  sign is the possible error in the determination due to a possible error of one minute in reading the angle of total reflection.

Expressing each of the above mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained:

"Insoluble" globulin.....	7.3	$\left\{ \begin{array}{c} \text{per cent} \\ 8.7 \\ 5.9 \end{array} \right\}$	$(\pm 0.4)$
Total globulins.....	28.0	$\left\{ \begin{array}{c} 31.0 \\ 22.0 \end{array} \right\}$	$(\pm 2.0)$
Total albumins.....	72.0	$\left\{ \begin{array}{c} 79.0 \\ 68.0 \end{array} \right\}$	$(\pm 2.0)$

The first figure opposite each group represents the average percentage; the upper figures immediately following, the highest percentage observed in any individual; the lower figure the lowest percentage observed in any individual; and the figure in parenthesis, the plus or minus error in the estimation of these percentages which would be brought about by an error of one minute in reading the angle of total reflection.

The following table indicates the results obtained with fasted pigeons.

TABLE II.  
*Fasted pigeons.*

GROUP NO.	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
4	$0.13 \pm 0.04$	$0.68 \pm 0.15$	$3.9 \pm 0.2$	$4.6 \pm 0.2$
5	$0.13 \pm 0.04$	$0.67 \pm 0.15$	$4.3 \pm 0.2$	$5.0 \pm 0.2$
Average	$0.13 \pm 0.04$	$0.67 \pm 0.15$	$4.1 \pm 0.2$	$4.8 \pm 0.2$

Expressing each of the above mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained:

"Insoluble" globulin.....	2.7	$\begin{Bmatrix} 2.8 \\ 2.6 \end{Bmatrix}$	( $\pm 0.4$ )
Total globulins.....	14.0	$\begin{Bmatrix} 15.0 \\ 13.0 \end{Bmatrix}$	( $\pm 2.0$ )
Total albumins.....	86.0	$\begin{Bmatrix} 86.0 \\ 85.0 \end{Bmatrix}$	( $\pm 2.0$ )

By comparison of Tables I and II, a decided increase of total albumins may be observed in the serum of the fasted animal. A similar result was obtained with rooster serum.

#### B. Rooster serum.

The following numbers indicate the percentage of the protein present in the original serum.

TABLE III.  
Normal roosters.

ROOSTER NO.	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	$0.53 \pm 0.04$	$1.34 \pm 0.15$	$2.0 \pm 0.2$	$3.3 \pm 0.2$
2	$0.56 \pm 0.04$	$1.16 \pm 0.15$	$2.0 \pm 0.2$	$3.2 \pm 0.2$
Average	$0.54 \pm 0.04$	$1.25 \pm 0.15$	$2.0 \pm 0.2$	$3.3 \pm 0.2$

Expressing each of the above mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained:

"Insoluble" globulin.....	16.4	$\begin{Bmatrix} 17.5 \\ 16.1 \end{Bmatrix}$	( $\pm 0.4$ )
Total globulins.....	38.0	$\begin{Bmatrix} 41.0 \\ 36.0 \end{Bmatrix}$	( $\pm 2.0$ )
Total albumins.....	62.0	$\begin{Bmatrix} 63.0 \\ 61.0 \end{Bmatrix}$	( $\pm 2.0$ )

With fasted roosters the following results were obtained:

## Proteins of Blood Sera

TABLE IV.  
*Fasted roosters.*

ROOSTER NO.	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
3		1.56 $\pm$ 0.15	3.0 $\pm$ 0.2	4.5 $\pm$ 0.2
4	0.57 $\pm$ 0.04	1.03 $\pm$ 0.15	3.1 $\pm$ 0.2	4.1 $\pm$ 0.2
5	0.49 $\pm$ 0.04	1.97 $\pm$ 0.15	2.8 $\pm$ 0.2	4.7 $\pm$ 0.2
6	0.43 $\pm$ 0.04	1.10 $\pm$ 0.15	2.6 $\pm$ 0.2	3.7 $\pm$ 0.2
Average	0.50 $\pm$ 0.04	1.41 $\pm$ 0.15	2.9 $\pm$ 0.2	4.13 $\pm$ 0.2

Expressing each of the above mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained:

"Insoluble" globulin.....	12.1	$\left\{ \begin{array}{l} 13.9 \\ 10.4 \end{array} \right\}$	( $\pm$ 0.4)
Total globulins.....	33.0	$\left\{ \begin{array}{l} 42.0 \\ 25.0 \end{array} \right\}$	( $\pm$ 2.0)
Total albumins.....	68.0	$\left\{ \begin{array}{l} 76.0 \\ 60.0 \end{array} \right\}$	( $\pm$ 2.0)

By comparison of the percentages of total albumins listed in Tables III and IV, it will again be seen, as in the case of the pigeon, that the fasted animals show an increase in total proteins.

Upon comparing these results with the data obtained by Thompson for fasted roosters,<sup>2</sup> it will be seen that the content of total albumins which is indicated by his results considerably exceeds that obtained by mine. In both series of experiments the length of the fast was approximately the same, but the birds employed by Thompson in obtaining his data were much older than those used in obtaining the above results. The former were all over four years of age, while the latter were all between the ages of one and two years. This difference in age may be the source of the markedly higher figures for the total albumins found by Thompson.

*C. Guinea fowl serum.*TABLE V.  
*Normal guinea fowls.*

GROUP NO.	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.23 $\pm$ 0.04	1.12 $\pm$ 0.15	2.6 $\pm$ 0.2	3.7 $\pm$ 0.2

<sup>2</sup> Compare the preceding article, p 1.

Expressing each of the above mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained:

	<i>per cent</i>
"Insoluble" globulin.....	6.2 $\pm$ 0.4
Total globulins.....	30.0 $\pm$ 2.0
Total albumins.....	70.0 $\pm$ 2.0

TABLE VI.  
*Fasted guinea fowls.*

GROUP NO.	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2	0.20 $\pm$ 0.04	0.22 $\pm$ 0.15	3.9 $\pm$ 0.2	4.1 $\pm$ 0.2

Expressing each of the above mentioned proteins in terms of the percentage of total proteins which they represent, the following figures are obtained:

	<i>per cent</i>
"Insoluble" globulin.....	4.7 $\pm$ 0.4
Total globulins.....	5.0 $\pm$ 2.0
Total albumins.....	95.0 $\pm$ 2.0

These results, as in the case of the pigeon and rooster, again show a decided increase in the total albumins of the serum of the fasted birds.

#### SUMMARY.

The following table summarizes the average results obtained.

		TABLE VII.		
		"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Pigeon.....	{ normal	7.3	28	72
	{ fasting	2.7	14	86
Rooster.....	{ normal	16.4	38	62
	{ fasting	12.1	33	68
Guinea fowl..	{ normal	6.2	30	70
	{ fasting	4.7	5	95





## PRECIPITATION OF SERUM-ALBUMIN AND GLUTIN BY ALKALOIDAL REAGENTS.

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The study of the mechanism of the precipitation of proteins by so called alkaloidal reagents (tannin, iodine potassium iodide, potassium ferrocyanide, potassium mercuric iodide, tungstate, and phosphomolybdate) has received practically no attention from the standpoint of physical chemistry, especially the isoelectric point. According to Michaelis,<sup>1</sup> the isoelectric point of serum is the point where the number of positive and negative charges of electricity held by the protein ions are about equal; that is, at this point there is a maximum of neutral particles of protein. This lies approximately at the concentration of  $2 \cdot 10^{-5}$  of acid, such as obtains in a mixture of equal parts ( $\frac{1}{2}$ ) of  $\frac{N}{10}$  acetic acid and  $\frac{N}{10}$  sodium acetate. Certain other phenomena which are exhibited by proteins at this point are, according to Pauli<sup>2</sup> and his pupils, a minimum of viscosity as shown by serum and a maximum of precipitation by ethyl alcohol. It would be interesting to know the behavior of other precipitants, such as the alkaloidal reagents, with respect to this point. It might also indicate whether the mechanism of precipitation by all alkaloidal reagents is the same or different. This has hitherto been regarded as identical.

In the following experiments attempts have been made to ascertain where precipitation of serum and glutin takes place with respect to their isoelectric points, and how it is influenced by different degrees of acidity and the addition of salt. The conditions under which precipitation was observed have been expressed in a quantitative manner; that is, definite quantities of serum and glutin of known protein content were mixed with definite quanti-

<sup>1</sup> L. Michaelis and B. Mostunski: *Biochem. Ztschr.*, xxiv, p. 79, 1910.

<sup>2</sup> W. Pauli: *Ztschr. f. Chem. u. Ind. d. Koll.*, xii, p. 222, 1913.

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ties of known strengths of acids, and to this the same quantity of the reagent of known strength was added. The total volume of the mixture was always the same. The effect of salt was studied in a similar manner.

All experiments were performed at ordinary room temperature in test-tubes. The precipitates were observed when freshly formed and at the end of twenty-four hours. However, it is to be noted that, as a rule, the precipitates remained unchanged on standing, and the data have been compiled without reference to this factor. Precipitation was regarded to have taken place when the mixture upon the addition of the reagent became non-transparent. The different degrees of precipitation have been expressed

TABLE I.\*

*Degree of acidity represented by acetic-acetate ("buffer") mixtures.*

	PROPORTION OF ACETIC ACID TO ACETATE IN MIXTURE: $\frac{N}{10}$ ACETIC ACID $\frac{N}{10}$ SODIUM ACETATE				
	$\frac{1}{1}$	$\frac{1}{2}$	$\frac{1}{3}$	$\frac{1}{4}$	$\frac{1}{5}$
[H <sup>+</sup> ]	$1.44 \times 10^{-4}$	$0.9 \times 10^{-4} \dagger$	$0.72 \times 10^{-4}$	$0.54 \times 10^{-4} \dagger$	$0.36 \times 10^{-4}$
P <sub>H</sub>	3.94	4.05 †	4.14	4.27 †	4.44 †
[H <sup>+</sup> ]	$0.27 \times 10^{-4} \dagger$	$1.8 \times 10^{-5}$	$0.9 \times 10^{-5}$	$0.45 \times 10^{-5}$	$0.22 \times 10^{-5}$
P <sub>H</sub>	4.57 †	4.74	5.05	5.35	5.66
					5.74 †

\*In the tables, the various signs and abbreviations used have meanings as follows: the plus sign (+) = precipitate present; the minus sign (−) = no precipitate; al = slight; st = strong; max = maximum; (?) = precipitate doubtful; tr = trace. The order in which the solutions were fixed and the quantities used are appended as footnotes to each table.

† Calculated.

in ordinary terms as indicated in the various tables. The reagents were previously rendered neutral to litmus by the addition of sodium hydroxide, and the strengths of these were usually 5 per cent. The horse serum and glutin used in these experiments had been previously dialyzed for a period of five to six weeks, and were practically salt-free. The end concentration represented in the different experiments will be found appended to each table. The standard "buffer" mixtures of acetic acid and sodium acetate were prepared according to Sørensen,<sup>3</sup> and the degrees of acidity that they represent are shown in Table I. In my work the concen-

<sup>3</sup> Sørensen: *Ergebn. d. Physiol.*, xii, p. 393, 1912.

tration of sodium acetate in all experiments is always the same, only the variation in the acetic acid being present. The different concentrations of acetic and hydrochloric acids were prepared by dilution of standard solutions in the usual manner, and the concentrations of these are indicated in the various tables.

*1. Precipitation of serum and gluten in mixtures of acetic acid and sodium acetate.*

In mixtures of acetic acid and sodium acetate with proteins, the dissociated hydrogen ions are not bound by the protein, but

TABLE II.  
*Precipitation of serum and gluten by alkaloidal reagents in the acetic-acetate mixtures.*

N TO ACETIC ACID	IODINE POTASSIUM IODIDE		POTASSIUM MERCURIC IODIDE, 5 PER CENT		SODIUM MOLYBDATE, 5 PER CENT		SODIUM PHOSPHO- TUNGSTATE, 5 PER CENT		POTASSIUM FERRO- CYANIDE, 5 PER CENT		TANNIN, 1 PER CENT	
N TO SODIUM ACETATE	S	G	S	G	S	G	S	G	S	G	S	G
5	-	-	+	+ st	+	+ st	+	+	+ st		-	-
4	+	-	+	+	+	+ less st	+	-	+ st		+ sl	+
3	+	-	+	+	+	+ least	+	-	+		+ sl	+
2	+	-	+	+	+ sl	-	+	-	+		+	+
1.5	-	-	+	+	+	-	max + sl	-	-			+
1	-	-	+ sl	+	+ ?	-	+ sl	-	-		+ st	+ st
1	-	-	+ sl		-	-	-	-	-		+	+
1	-	-	-		-	-	-	-	-		+ ?	-
1	-	-	-		-	-	-	-	-		-	-
Without acid	-	-	-	-	-	-	-	-	-		-	-

"S" refers to horse serum; "G" to gluten. Order of mixing solutions: acetic-acetate mixture 1 cc., water 1 cc., reagent 1 drop and serum (0.5 per cent) or gluten (0.5 per cent) 1 drop.

are made available for the precipitation reaction with the alkaloidal reagents. It is thus possible to ascertain if precipitation is concerned (1) with neutral particles of protein, (2) with positive protein ions, or (3) whether it depends upon the liberation of the free acid of the reagents. With this in view, experiments with horse serum and gluten were made according to the method described above, the details of which are appended to the results presented in Table II.

From this it is to be seen that the precipitation of serum by the following reagents, iodine potassium iodide, potassium mercuric iodide, potassium ferrocyanide, sodium tungstate, and phos-

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phomolybdate, takes place above the isoelectric point, and in the direction of higher concentrations of acetic acid, and that it increases as the acidity increases. Precipitation by the different reagents begins practically with the same mixture of acid and acetate, and the small variations which occur are probably within experimental error. Inasmuch as the dissociated acid is not bound to the protein ion under these conditions, it would seem to indicate that a certain amount of free and excess of acid is necessary for the formation of the insoluble protein compounds.

On the other hand, with tannin, the maximum of precipitation occurs at about the isoelectric point, and then diminishes on either side of it; that is, with either increased or decreased concentrations of free acid. This would indicate that the mechanism of precipitation by tannin is different from that of the other alkaloidal reagents used.

With glutin, precipitation takes practically the same course as with serum. No precipitation was observed with iodine and potassium iodide and the ferrocyanide. Tannin exhibits practically the same differences from the other reagents as with serum.

### *2. Precipitation of serum and glutin by alcohols in mixtures of acetic acid and acetate.*

From the preceding section it appears that the mechanism of the precipitation of proteins by tannin is different from that with the other reagents. It is possible that the mechanism is similar to that of certain alcohols; for ethyl alcohol also produces a maximum of precipitation at the isoelectric point. This analogy was tested out by observing the precipitation of serum and glutin by different alcohols with respect to the isoelectric point. The data are presented in Table III.

It is seen that the maximum of precipitation of serum by the different alcohols used occurs at about the isoelectric point; *i.e.*, with mixtures of one to two ( $\frac{1}{2}$ ) and one to four ( $\frac{1}{4}$ ) parts of acetic acid and acetate, respectively. With the exception of propyl alcohol, the precipitates diminish in intensity on either side of this point. There appeared to be no difference in the precipitates produced by propyl alcohol. It is also to be noted that

TABLE III.

*Precipitation of serum by alcohols in the acetic-acetate mixtures.*

N/10 ACETIC ACID N/10 SODIUM ACETATE	PHENOL, 5 PER CENT	RESORCIN, 5 PER CENT	HYDROQUINONE, 5 PER CENT	PROPYL ALCOHOL
2	—	—	—	—
1.5	—	—	—	—
1	—	—	—	—
0.5	+	+	—	—
0.25	+	+	+ st	+ tr
0.125	+ st	+ st	+ st	+ st
0.0625	+ st	+ st	+ less st	+ st
0.03125	+ less st	+ less st	+ still less st	+ st
0.015625	+ still less st	+ least st	+ tr	+ st
Serum and water (equal parts)	+ least st	+ least st	+ tr	—

Order of mixing solutions: horse serum (0.5 per cent) 1 cc., acetic-acetate mixture 1 cc., and alcohol reagent 1 cc.

serum alone without the acid-acetate mixture gave slight precipitates with all alcohols except propyl alcohol. The data strongly indicate that the mechanism of precipitation by tannin more closely resembles the alcohols than the other alkaloidal reagents.

The results obtained with glutin (Table IV) lead to practically the same conclusions.

TABLE IV.

*Precipitation of glutin by alcohols in mixtures of acetic acid and acetate.*

N/10 ACETIC ACID N/10 SODIUM ACETATE	PHENOL, 5 PER CENT	RESORCIN, 5 PER CENT	HYDROQUINONE, 5 PER CENT	PROPYL ALCOHOL
2	—	+ less st	—	+
1.5	+	+ st	+	+
1	+	+ st	+	+
0.5	—	+ v. sl	—	+
0.25	—	—	—	—
0.125	—	—	—	—
0.0625	—	—	—	—
0.03125	—	—	—	—

Order of mixing solutions: glutin (0.5 per cent) 0.5 cc., acetic-acetate mixture 0.5 cc., water 1 cc., and alcohol 2 cc.

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### 3. *Precipitation of serum and glutin in acids.*

Thus far it is seen that the precipitation of serum and glutin by the various reagents occurs when acid protein is present; that is, it takes place in the presence of a concentration of hydrogen ions higher than the isoelectric point, and, apparently, is augmented when these are in excess. However, it still remains to be shown whether precipitation depends upon the liberation of the acid of the reagent, or the formation of protein salts with added acid and the reaction of these with the reagent.

According to Pauli, the dissociated hydrogen ions of such acids as acetic or hydrochloric are bound by protein with the formation of dissociated protein ions with positive electric charges. The number of these charges increases with the addition of more acid until all of the protein is saturated. This occurs even in the lowest concentrations of acid. Such bound hydrogen ions do not become available for other reactions unless the quantity of protein remains constant and an excess of acid is added. If, then, precipitation in a mixture of protein and acid by a reagent occurs only during the phase of excess acid and not when protein ions only are present, the conclusion must be drawn that precipitation depends upon the presence of free acid and not upon dissociated protein ions alone; that is, the precipitate is a combination of the free acid of the salt with the protein or acid protein. This, indeed, is the case with iodine potassium iodide, mercuric iodide, ferrocyanide, tungstate, and phosphomolybdate, and to a certain extent with tannin.

The experiments were performed by adding small and constant quantities of serum and glutin to different concentrations of acetic and hydrochloric acids and noting where precipitation occurred by the further addition of constant quantities of different reagents. The data obtained, as well as the details in which the experiments were performed, are presented in Table V.

It is seen that in the lowest concentrations of both acids, where dissociated protein ions occur, no precipitation of serum with any of the reagents took place. Precipitation occurred only with the higher concentrations; that is, in the presence of an excess of acid. The beginning of precipitation with the different reagents varies, and small but negligible variations are to be noted in the same reagent with different acids. With tannin, a maximum of pre-

TABLE V.

*Precipitation of serum and glutin by alkaloidal reagents in dilute acids.*

END CON- CENTRATION OF ACID	IODINE IN POTASSIUM IODIDE		POTASSIUM MERCURIC IODIDE, 5 PER CENT		SODIUM PHOSPHOMO- LYBDATE, 5 PER CENT		SODIUM PHOSPHO- TUNGSTATE, 5 PER CENT		POTASSIUM FERRO- CYANIDE, 5 PER CENT		TANNIN, 1 PER CENT	
	CH <sub>3</sub> CO <sub>2</sub> H	HCl	CH <sub>3</sub> CO <sub>2</sub> H	HCl	CH <sub>3</sub> CO <sub>2</sub> H	HCl	CH <sub>3</sub> CO <sub>2</sub> H	HCl	CH <sub>3</sub> CO <sub>2</sub> H	HCl	CH <sub>3</sub> CO <sub>2</sub> H	HCl
<i>Serum.</i>												
5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-
7.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-
10.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	+ sl	-
1.5.10 <sup>-3</sup>	+ ?	-	+	+	-	-	-	-	-	-	+	+ ?
2.5.10 <sup>-3</sup>	+ sl	-	+	+	+	-	-	-	-	-	+ st	+
4.10 <sup>-3</sup>	+ sl	+ sl	+	+	+	-	-	-	-	-	+ st	+ ?
5.10 <sup>-3</sup>	+ ?	+ sl	+	+	+	-	-	-	-	-	+less st	+ ?
10.10 <sup>-3</sup>	-	+	+st	+	+	-	-	-	-	+ sl	+less st	-
1.25.10 <sup>-2</sup>	-	-	+	+	+	+ sl	+ sl	-	-	+ sl	+	-
2.5.10 <sup>-2</sup>	-	-	+	+	+	+	+ sl	-	+ sl	+less st	+	-
5.10 <sup>-2</sup>	-	-	+	+	+	+	+ st	+	+	+ st	+least	-
<i>Glutin.</i>												
7.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-
10.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-
1.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-
2.5.10 <sup>-3</sup>	-	-	-	+	-	-	-	-	-	-	-	+
5.10 <sup>-3</sup>	-	-	+	+	-	-	-	-	-	-	+ sl	+
10.10 <sup>-3</sup>	-	-	+	+	-	-	-	-	-	-	+more	+
1.25.10 <sup>-2</sup>	-	-	+	+	-	+ sl	-	-	-	-	+ still	+
2.5.10 <sup>-2</sup>	-	-	+	+	+ sl	+ sl	-	-	-	-	more	+
5.10 <sup>-2</sup>	-	-	+	+	+ sl	+more	-	+ sl	+ sl	+ sl	+ still more	+

Order of mixing solutions: acid 1 cc., water 1 cc., glutin or horse serum (0.5 per cent) 1 cc., and reagent 1 drop.

precipitation in both acids occurred at the concentration of  $2.5 \cdot 10^{-4}$ . In the higher concentrations the precipitates dissolved. This was also true of iodine and potassium iodide, which at no time gave absolutely distinct precipitates. This phenomenon perhaps depends upon the formation of soluble acid salts and requires further investigation.

With glutin, practically the same results were obtained as with serum.



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It is to be concluded that the precipitation of serum and glutin in hydrochloric and acetic acids by iodine potassium iodide, mercuric iodide, ferrocyanide, tungstate, and phosphomolybdate, takes place only in the presence of an excess of free acid and depends upon the formation of insoluble compounds with the free acid of the salt.

### *4. The effect of salts on the precipitation of serum and glutin by alkaloidal reagents.*

The addition of salt to an acid might conceivably alter the precipitation reaction by alkaloidal reagents and alcohols. This was studied in the following manner: To constant and definite concentrations of glutin and serum in the same volumes of acetic and hydrochloric acids of different concentrations were added constant volumes of potassium chloride and potassium sulphocyanide, and finally to this mixture constant and definite quantities of the reagents. Each series of experiments with the different salts was compared with a series without salt; that is, with distilled water as a blank. The results from all of the experiments are to be found in Tables VI, VII, and VIII.

From these data it is seen that the two salts, chloride and sulphocyanide, exerted practically no influence upon the formation of the precipitate by the various reagents used. Practically no precipitation occurred in any case until the concentration of  $4 \cdot 10^{-4}$  was reached. The small variations which occurred fall within the experimental error. This was true of both serum and glutin.

With tannin, precipitation in the presence of the chloride and sulphocyanide occurred in the lowest concentrations of the hydrochloric acid and before  $4 \cdot 10^{-4}$ . Thus a small difference from the other reagents is indicated.

With alcohols, the addition of salts also had practically no influence upon the precipitation. The slight variations exhibited by the sulphocyanide are too small to permit the drawing of any conclusions. The same can be said of glutin in acetic acid-acetate mixtures (Table IX).

TABLE VI.  
Effect of salts on the precipitation of serum by alkaloidal reagents.

END CON- CENTRATION OF HCl	IODINE IN POTASSIUM IODIDE			POTASSIUM MERCURIC IODIDE, 5 PER CENT			SODIUM PHOSPHO- MOLYPDATE, 5 PER CENT			SODIUM PHOS- PHOTUNGSTATE, 5 PER CENT			POTASSIUM FERROCYANIDE, 5 PER CENT			TANNIN, 1 PER CENT		
	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN
7.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
1.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
2.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
4.10 <sup>-4</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.10 <sup>-4</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10.10 <sup>-4</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.25.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
2.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
ACETIC ACID																		
7.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1.5.10 <sup>-4</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.5.10 <sup>-4</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4.10 <sup>-4</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.10 <sup>-4</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
1.25.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
2.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+

Order of mixing solutions: acid 1 cc.,  $\frac{N}{10}$  salt solution (KCl or KSCN) 1 cc., horse serum (0.5 per cent) 1 drop, and reagent 1 drop.

TABLE VII  
Effect of salts on the precipitation of glutin by alkaloidal reagents.

END CON- CENTRATION OF HCl	IODINE IN POTASSIUM IODIDE			POTASSIUM MERCURIC IODIDE, 5 PER CENT			SODIUM PICO- PRONATE, 5 PER CENT			POTASSIUM PERBICARBONATE, 5 PER CENT			TANNIN, 1 PER CENT		
	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN
2.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
7.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
10.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
10.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1.25.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ACETIC ACID															
2.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
7.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
10.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
10.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1.25.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2.5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
5.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Order of mixing solutions: acid 1 cc., water or  $\frac{N}{10}$  salt (KCl or KSCN) solution 1 cc., glutin (0.5 per cent) 1 drop, and reagent 1 drop.

TABLE VIII.

*Effect of salts on the precipitation of serum and gluten by alcohols.*

END CONCENTRATION OF HCl	PHENOL, 5 PER CENT			RESORCIN, 5 PER CENT			HYDROQUINONE, 5 PER CENT			PROPYL ALCOHOL		
	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN	H <sub>2</sub> O	KCl	KSCN
1.25.10 <sup>-3</sup>	-		-	-		-	-	-	-	-	-	-
0.2.10 <sup>-4</sup>	-		-	-		-	-	-	-	-	-	+ sl
0.3.10 <sup>-4</sup>	-		-	-		-	-	-	-	-	-	+ sl
0.4.10 <sup>-4</sup>	-	+ sl	-	+ sl	+ sl	-	+?	-	-	+ sl	+ sl	-
1.25.10 <sup>-4</sup>	+	+ st	-	+ st	+ st	-	+	+	-	+ st	+ st	-
0.62.10 <sup>-3</sup>	-	+ al	+	-	+ al	+	-	+	+	-	+?	-
1.25.10 <sup>-3</sup>	-	-	+	-	-	+	-	-	+	-	-	-
0.62.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-
1.25.10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-	-	-	-
0.62.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-
1.25.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	-
ACETIC ACID												
0.5.10 <sup>-4</sup>	-		-	-		-	-		-	-		-
1.25.10 <sup>-4</sup>	-		-	+ sl		+ st	-		+	-		-
0.62.10 <sup>-3</sup>	+ sl		+?	+		+ st	+		+ st	+		+
1.25.10 <sup>-3</sup>	+		+ st	-		+ sl	-		-	-		-
0.62.10 <sup>-3</sup>	-		+ al	-		-	-		-	-		-
1.25.10 <sup>-3</sup>	-		+	-		-	-		-	-		-
0.62.10 <sup>-4</sup>	-		-	-		-	-		-	-		-
1.25.10 <sup>-4</sup>	-		-	-		-	-		-	-		-

Order of mixing solutions: acid 0.5 cc., gluten (0.5 per cent) 0.5 cc., water or N/5 salt (KCl or KSCN) solution 1 cc., and alcohol reagent 2 cc.

TABLE IX.

*Effect of sulphocyanide on the precipitation of gluten by alcohols in acetic-acetate mixtures.*

N/10 ACETIC ACID	RESORCIN, 5 PER CENT		PHENOL, 5 PER CENT		HYDROQUINONE, 5 PER CENT		PROPYL ALCOHOL	
N/10 SODIUM ACETATE	H <sub>2</sub> O	KSCN	H <sub>2</sub> O	KSCN	H <sub>2</sub> O	KSCN	H <sub>2</sub> O	KSCN
.....	+ sl	+ st	-	+	-	+	+	-
.....	+ st	+ st	+ st	+	+ st	+ sl	+ st	+?
.....	+ st	+ sl	+ st	-	-	-	+ st	+?
.....	+ sl	-	-	-	-	-	+	+?
.....	-	-	-	-	-	-	-	+?
.....	-	-	-	-	-	-	-	+?

Order of mixing solutions: 2 per cent gluten (1:4) 0.5 cc., acetic-acetate mixture 0.5 cc.,  $\frac{N}{5}$  KSCN 1 cc., and alcohol 2 cc. The end concentration of KSCN is  $\frac{N}{50}$ ; of gluten 0.5 per cent.

## 24 Precipitation of Serum-Albumin and Glutin

### 5. Effect of concentration of protein on the precipitation.

This was tested out by using two different concentrations of serum, 0.017 per cent and 1 per cent (as end concentrations of protein) in constant volumes of hydrochloric acid of different strengths and precipitated by the further addition of one drop of the reagent. The data have been placed in Table X.

As the results with the different concentrations of serum are practically identical, it is to be concluded that wide differences in protein content have no marked influence on the precipitation.

TABLE X.  
*Effect of concentration of serum-albumin on the precipitation by alkaloidal reagents.*

END CONCENTRATION OF HCl	IODINE POTASSIUM IODIDE		POTASSIUM MERCURIC IODIDE, 5 PER CENT		SODIUM MOLYBD- ATE, 5 PER CENT		SODIUM PHOSPHO- TUNGSTATE, 5 PER CENT		POTASSIUM FERRO- CYANIDE, 5 PER CENT		TANNIN, 1 PER CENT	
	Serum-albumin.											
	0.017 %	1 %	0.017 %	1 %	0.017 %	1 %	0.017 %	1 %	0.017 %	1 %	0.017 %	1 %
7.5.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	-	+
10.10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-	-	+ sl	+
1.5.10 <sup>-3</sup>	-	-	+	+	-	-	-	-	-	-	+	+
2.5.10 <sup>-3</sup>	-	-	+	+	-	-	-	-	-	-	+ st	+
5.10 <sup>-3</sup>	+	+ sl	+	+ sl	-	-	-	-	-	-	+ st	+
10.10 <sup>-3</sup>	+	+	+	+ st	-	-	-	-	+ sl	-	+ least st	+
1.3.10 <sup>-2</sup>	+	+	+	+ st	+ sl	-	-	+ sl	+ sl	+ sl	+ less st	-
2.5.10 <sup>-2</sup>	+	+	+	+ st	+ sl	+ sl	+	+ st	+	+	+ least st	-
5.10 <sup>-2</sup>	-	-	+	+ st	+ st	+ st	+	+ st max	+	+	-	-

Order of mixing solutions: horse serum 1 cc., acid 1 cc., and reagent 1 drop. The percentages under "serum-albumin" represent end concentrations of albumin.

### SUMMARY.

The mechanism of the precipitation of dialyzed horse serum and glutin by tannin is different from that of certain precipitants commonly known as "alkaloidal reagents." With these a certain amount of free acid (hydrogen ion concentration) is necessary for the formation of the complex insoluble compounds.

Tannin behaves like certain alcohols, *e.g.*, resorcin, phenol, hydroquinone, and propyl alcohol, since the maximum of precipitation in both cases corresponds to the isoelectric point in serum-albumin and glutin.

Precipitation of serum is uninfluenced by wide differences of concentration and the addition of such neutral salts as chloride and sulphocyanide.

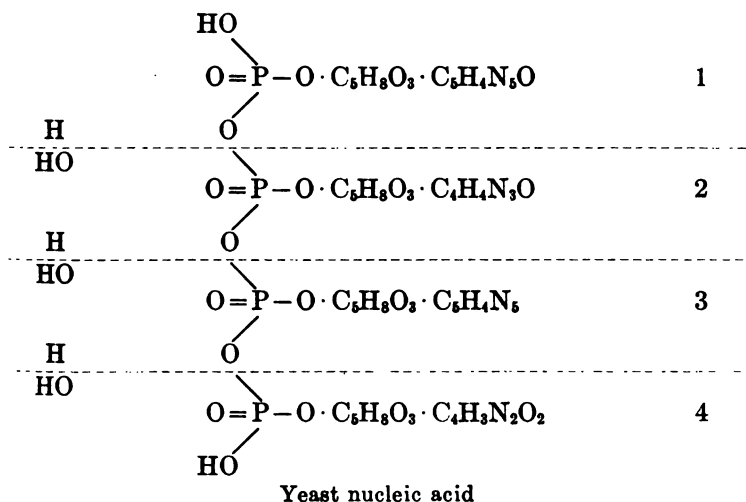
# SIMPLER NUCLEOTIDES FROM YEAST NUCLEIC ACID.

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It has been shown by Levene and Jacobs<sup>1</sup> that when yeast nucleic acid is boiled with dilute mineral acids, the tetra-nucleotide is decomposed into its four component mono-nucleotides, as is shown in the following diagram.

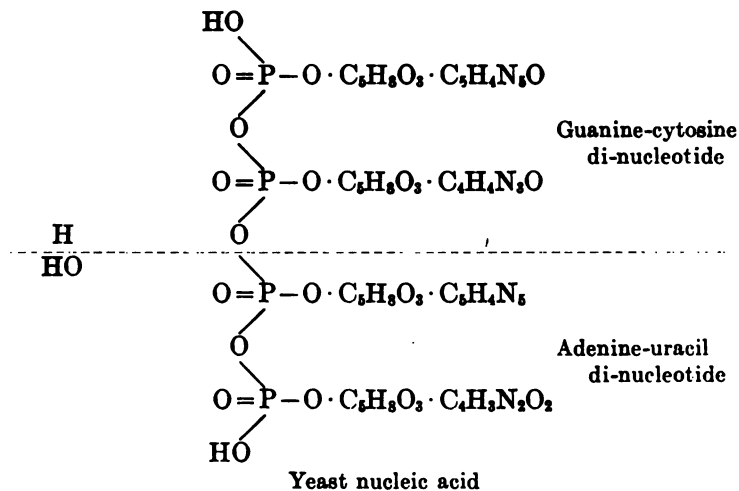


But at the same time the two purine mono-nucleotides (1 and 3) are further decomposed by the boiling mineral acid into purine bases, phosphoric acid, and pentose, while the two pyrimidine mono-nucleotides (2 and 4) are unaltered and may be obtained as

<sup>1</sup> P. A. Levene and W. A. Jacobs: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 1027, 1911.

end products of the hydrolysis. This is the first recorded instance of the formation of simpler nucleotides from a tetra-nucleotide, and the results furnish a method by which one can easily decide whether in a given instance he is dealing with a purine nucleotide, a pyrimidine nucleotide, or a mixed nucleotide containing both a purine and a pyrimidine group; for in such a case it is not necessary to make a tedious purine-pyrimidine separation, but is sufficient to estimate the phosphoric acid liberated by hydrolysis with dilute mineral acid.

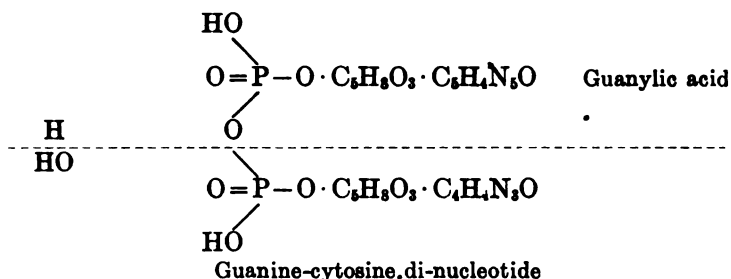
In a former contribution<sup>2</sup> we stated that the first stage in the enzymatic hydrolysis of yeast nucleic acid consists of a decomposition of the tetra-nucleotide into two di-nucleotides, as is shown in the following diagram.



A brief statement of the crucial properties of the di-nucleotides was made; *viz.*, that by acid hydrolysis the one yields essentially guanine and cytosine, while the other yields adenine and uracil; but full discussion of the substances was postponed. We showed that when the enzymatic hydrolysis of yeast nucleic acid proceeds farther, the guanine-cytosine di-nucleotide is decomposed into its component mono-nucleotides. One of these was found among the end products of the action of yeast on yeast nucleic acid and

<sup>2</sup> W. Jones and A. E. Richards: this *Journal*, xvii, p. 71, 1914.

was shown to be identical with the guanylic acid which is widely present in animal glands, but especially in the pancreas.



Since the appearance of our former paper the field has been considerably broadened by an important communication of Thannhauser,<sup>3</sup> who found that yeast nucleic acid is stripped of one of its nucleotide groups by the digestive action of duodenal juice, and there is formed a tri-nucleotide which contains groups of guanine, adenine, and cytosine, but not uracil. Thus six simpler nucleotides have been prepared from yeast nucleic acid,—a tri-nucleotide, two di-nucleotides, and three mono-nucleotides.

It is our purpose to discuss the two di-nucleotides and guanylic acid in this paper.

The extent to which enzymatic hydrolysis of yeast nucleic acid will proceed depends upon a number of factors. Various animal and plant tissues differ greatly from one another in the ferments which they contain, so that, under precisely the same conditions, extracts of different tissues will produce different end products by their action on the nucleic acid. But the same tissue extract may produce quite different end products by a change of the conditions under which its enzymatic activity is exerted. One of the principal factors in this connection is the relative amount of nucleic acid that is initially brought into contact with the ferment solution. Thus an aqueous extract of pig's pancreas by its action on nucleic acid may be made to yield either guanylic acid, guanosine, guanine, or xanthine, by diminishing successively the amount of nucleic acid employed with a constant amount of the pancreatic extract. Finally, the enzymatic decomposition of nucleic acid is brought

<sup>3</sup> S. J. Thannhauser: *Ztschr. f. physiol. Chem.*, xci, p. 329, 1914.



about by the successive action of a large number of independent agents or enzymes which differ markedly from one another in their stability at the body temperature, so that, by submitting a tissue extract to digestion at 40° certain of its ferments may be destroyed, while others remain in considerable activity, and therefore the end products of the action of such a predigested extract upon nucleic acid will be different from those of a fresh extract of the same tissue.

Advantage has been taken of this last circumstance in the preparation of the two di-nucleotides from yeast nucleic acid. If a fresh aqueous extract of pig's pancreas be allowed to act on yeast nucleic acid under the conditions stated below, the end products (as far as concerns the guanine group of the nucleic acid) will be guanylic acid and guanosine. The two substances will combine with one another to form an insoluble compound whose separation from the other products is easily accomplished by the method described in connection with yeast.<sup>4</sup> This decomposition of nucleic acid evidently proceeds with the formation in turn of:

1. Guanine-cytosine di-nucleotide.
2. Guanylic acid.
3. Guanosine.

For if the pancreatic extract be digested at 40° before adding the yeast nucleic acid, the ferments that decompose the di-nucleotides are destroyed, while the ferment that produces them from nucleic acid remains active, so that the two di-nucleotides compose the principal end product of the action of this extract on yeast nucleic acid.

These two substances have general properties somewhat similar to those of yeast nucleic acid, and like yeast nucleic acid they easily undergo decomposition when submitted to processes intended for their purification. It should not therefore be expected that they have been prepared in perfectly pure condition; but if the very fair approach of their chemical properties to theoretical requirements be taken into consideration with their origin from yeast nucleic acid, the structure of the compounds follows with considerable certainty.

<sup>4</sup> Jones and Richards: *loc. cit.*

*Preparation of the two di-nucleotides from yeast nucleic acid.*

A mixture of 2 kilos of carefully trimmed and ground pig's pancreas, 2 liters of water, and 30 cc. of chloroform was allowed to digest for twelve hours at the room temperature in a tightly closed vessel with frequent and violent agitation. After the tissue had by this means become thoroughly penetrated with chloroform, the mixture was placed in a thermostat and allowed to digest at 40° for two weeks, when it was cooled and filtered. The clear, pale yellow filtrate was then treated with yeast nucleic acid (1.5 grams for each 100 cc. of fluid) and again digested at 40° for twelve hours, when the complete disappearance of the nucleic acid was apparent from the failure of the clear fluid to produce a precipitate or even a cloud with sulphuric acid. The product was heated to boiling, filtered from a small coagulum, and treated at the boiling point with a 25 per cent solution of lead acetate as long as the reagent produced a precipitate in the hot fluid; but care was taken to avoid a great excess of lead acetate, which would precipitate the di-nucleotides. It is not at all difficult to make this precipitation accurately, because there is a wide margin between the point where the precipitation of lead phosphate, coloring matter, etc., ends, and the point where the precipitation of the di-nucleotides begins. A number of objectionable substances are thus removed, leaving a clear, pale yellow fluid which can easily be filtered from the bulky lead precipitate, and which forms a heavy, granular precipitate when treated at the boiling point with an excess of lead acetate. This lead precipitate increases in amount as the fluid cools, and settles so rapidly that the supernatant liquid may be removed by decantation. It consists of a mixture of the lead salts of the two di-nucleotides. By suspending the lead salts in warm water and treating with hydrogen sulphide, a solution of the free di-nucleotides is obtained.

The method of separating the two substances from one another is based upon the peculiar conduct of their potassium salts when treated in aqueous solution with an excess of alcohol, the one substance being immediately precipitated, while the other remains emulsified. Accordingly, the aqueous solution of the di-nucleotides was filtered from lead sulphide, concentrated under diminished pressure at 45°, and treated warm with a small amount of

hot concentrated potassium acetate. The potassium salts are slowly formed and the solution becomes gelatinous as it cools, but before gelatinization has proceeded far, the warm aqueous solution is stirred into an excess of alcohol. The guanine-cytosine di-nucleotide is immediately thrown out as a perfectly white flocculent precipitate, which falls rapidly, while the adenine-uracil di-nucleotide remains suspended for a long time, but on standing over night becomes deposited as a resin leaving a perfectly transparent fluid. This resin which entraps all the coloring matter of the solution may be hardened by treatment with absolute alcohol and finally ground to a pale yellow, dry powder.

It would be surprising if this process should effect a sharp separation of the two substances and one might expect the properties of each compound to be expressed in the other. Nevertheless, the separation is sufficiently accurate to establish the most important property of both compounds; *i. e.*, that each yields principally but one purine derivative and but one pyrimidine derivative.

*The guanine-cytosine di-nucleotide.*

The crude substance obtained as described and dried with absolute alcohol was made into a hot concentrated solution and treated with an equal volume of glacial acetic acid which produces a bulky gelatinous precipitate. This was filtered off and the filtrate was poured into a large volume of glacial acetic acid. The precipitated di-nucleotide was filtered off as closely as possible with a pump, dissolved in a little hot water, and after the addition of a small amount of potassium acetate was poured into a large excess of alcohol. The precipitated di-nucleotide was rapidly and roughly washed free from the cloudy solution by decantation with absolute alcohol, dried in a desiccator with sulphuric acid, and finally heated to a constant weight at 105°. The substance was thus obtained as a perfectly white non-hygroscopic powder, easily soluble in water and laevorotatory to polarized light.

Two grams dissolved in 50 cc. of water gave a reading of  $-1^{\circ}36'$  in a 2 dm. tube ( $\alpha_D = -20^{\circ}$ ).

Yeast nucleic acid, on the contrary, is strongly dextrorotatory. Analysis of three different preparations of the di-nucleotide gave the following percentages:

	FOUND			REQUIRED FOR $C_{28}H_{24}P_2N_8O_{11}K_2$
	I	II	III	
Potassium.....	9.9	10.2	10.4	10.8
Nitrogen.....	15.2	15.6	15.5	15.0
Phosphorus.....	7.8	7.8	7.7	8.3

Several weighed portions of the di-nucleotide, 1 gram each, were heated with 10 cc. of 5 per cent sulphuric acid for an hour in a small flask provided with a simple condensing tube and immersed in boiling water. The product while still hot was treated with an excess of ammonia, and, after cooling, the coarsely granular guanine was filtered off, dried, and weighed. The ammoniacal filtrates were then treated with magnesia mixture for the precipitation of phosphoric acid, and the filtrates from magnesium ammonium phosphate were tested for purine bases with an ammoniacal solution of silver nitrate. In case a small precipitate formed it was decomposed and tested for adenine.

The specimens of magnesium ammonium phosphate were dissolved in nitric acid and precipitated in turn as ammonium phosphomolybdate and ammonium magnesium phosphate. The latter were weighed and the phosphorus was calculated. It will be seen from the results tabulated below that only about half the total phosphoric acid of the compound is liberated thus by mild acid hydrolysis, which shows that the substance is a mixed di-nucleotide containing both a purine and a pyrimidine group.

Each specimen of guanine, after weighing, was converted into the chloride which consisted uniformly of the feathery, macroscopic needles characteristic of guanine chloride. Some of the specimens were analyzed.

The results of five such experiments made with different preparations are expressed as percentages in the following table.

	GUANINE			ADENINE	PHOSPHORUS		
	Theoretical	Found	Theoretical for yeast nucleic acid		Total	Half	Found
I....	20.3	21.7	10.5	0	8.3	4.2	3.2
II....		20.9		0			3.4
III....		21.3		Trace			3.3
IV....		22.0		0			3.6
V....		21.1		Trace			4.0

Thirty grams of the di-nucleotide were heated for three hours with 25 per cent sulphuric acid in an autoclave at 150°, and the product was examined for pyrimidine derivatives by the well known silver method. Four and a half grams of characteristic cytosine picrate were obtained, from which the free base was prepared, dried at 115°, and analyzed (N=37.42 and 37.61, instead of 37.85). Uracil could not with certainty be identified among the products. A trace of a pyrimidine derivative was obtained out of the filtrate from cytosine picrate, but it could not be determined whether it was cytosine or uracil. Its amount was too small to be of any significance.

*The adenine-uracil di-nucleotide.*

The crude pale yellow powder was purified by means of glacial acetic acid as described above; but the substance could not be obtained in as pure a condition as the di-nucleotide discussed in the previous section. Its decomposition products showed it to be contaminated with considerable traces of this substance, as might have been expected from the crude, but only available method of separating the two substances from one another. But this di-nucleotide fulfils its two most necessary requirements. It liberates approximately one-half of its phosphoric acid by mild acid hydrolysis, and yields nearly twice as much uracil as can be obtained from a corresponding amount of yeast nucleic acid. From 35 grams of the substance by hydrolysis with 25 per cent sulphuric acid in the autoclave at 150°, there were obtained 4.08 grams of pure recrystallized uracil in characteristic needle clusters. (N=25.01, 25.11, 25.07, 25.03, instead of 25.00.)

The di-nucleotide is slightly laevorotatory to polarized light. Two grams of substance in 50 cc. of water gave a reading of  $-36'$  in a 2 dm. tube  $(\alpha)_D = -7.5^\circ$ .

A number of specimens of the di-nucleotide, 1 gram each, were heated on the water bath for an hour with 5 per cent sulphuric acid. The hot solution was made strongly alkaline with ammonia, and the small amount of guanine which was deposited when the solution cooled was filtered off. The filtrate was then examined for adenine and free phosphoric acid. The results of five such experiments are expressed as percentages in the following table.

	GUANINE		ADENINE			PHOSPHORUS		
	Theoretical	Found	Theoretical	Found	Theoretical for yeast nucleic acid	Total	Half	Found
I.....	0	1.2	20.4	13.7	9.6	9.37	4.68	4.01
II.....		1.0		15.2				4.12
III.....		0.8		12.8				4.13
IV.....		0.2		14.6				3.96
V.....		1.1		14.7				4.21

These results show that the material was somewhat contaminated with the guanine-cytosine di-nucleotide, or with some undecomposed nucleic acid. But the low values for adenine are of little significance; for no better results for adenine can be obtained with the purest preparations of yeast nucleic acid.<sup>5</sup> It is probable that the analytical procedure for adenine is not as exact as is commonly supposed.

#### *Guanylic acid.*

In our former contribution we showed how yeast nucleic acid is decomposed by the ferments of yeast with the production of a compound of guanylic acid and guanosine whose difficult solubility makes it easily accessible. From this substance (which is formed also by the action of fresh pancreas extract on yeast nucleic acid) guanylic acid may best be prepared in pure condition by the following procedure. The substance is dissolved in a comparatively

<sup>5</sup> See results of P. A. Levene: *Biochem. Ztschr.*, xvii, p. 120, 1909.

large amount of hot water and treated with a solution of lead acetate. To prevent any guanosine from being carried down with the heavy granular lead guanylate, the precipitation is made slowly and at the boiling point. After thorough washing with boiling water the lead compound is suspended in cold water and decomposed with hydrogen sulphide. The excess of the reagent is driven out of the cold solution by a current of air and the fluid is evaporated to a small volume at 50° under diminished pressure. Upon cooling in ice water, perfectly white guanylic acid is deposited. This sometimes contains a trace of guanosine which may be removed by dissolving the guanylic acid in cold water (which leaves the guanosine undissolved) and precipitating the material with alcohol. A very pure product may also be obtained by the addition of alcohol to a mother-liquor from which a little guanylic acid has been deposited. Analysis of seven different preparations precipitated with alcohol and dried at 105° gave the following results:

- I. 0.2783 required 14.58 cc. of standard acid (1 cc. = 0.003695 N).
- II. 0.1614 required 8.24 cc.
- III. 0.2118 required 10.88 cc.
- IV. 0.5351 gave 0.3498  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$
- V. 0.5415 gave 0.3571  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$
- VI. 0.5238 gave 0.3412  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$
- VII. 0.4644 gave 0.3000  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$

	FOUND							THEORETICAL FOR $\text{C}_{10}\text{H}_{16}\text{N}_4\text{P}_2\text{O}_{13}$
	I	II	III	IV	V	VI	VII	
N .....	19.35	18.86	18.98					19.28
P .....				8.36	8.37	8.27	8.51	8.55

The formation of guanylic acid from yeast nucleic acid suggests the explanation of an interesting and curious matter. The tetra-nucleotide which is a constituent of animal cell nuclei, *i.e.*, the so called animal nucleic acid, contains a hexose group. This can scarcely be doubted, for by severe acid hydrolysis the substance yields both formic acid and laevulinic acid, and by oxidation with nitric acid it forms one of the saccharic acids. On the other hand, the tetra-nucleotide of plant cell nuclei or plant nucleic acid possesses a pentose group, and pentose is formed when the substance

is hydrolyzed. Guanylic acid is a mono-nucleotide present in animal glands and might be expected to resemble in its chemical structure the tetra-nucleotide of animal tissues. But this is not the case. Guanylic acid contains a pentose group, not a hexose group, and by acid hydrolysis it forms not only a pentose but the particular pentose which is formed similarly from plant nucleic acid (d-ribose).<sup>6</sup> The idea is at once suggested that the guanylic acid of animal tissues is formed from the nucleic acid of plant food, and this idea is strongly supported by the actual production of guanylic acid from yeast nucleic acid by the action of ferments.

In order to throw additional light upon this question we undertook the quantitative preparation of guanylic acid from the pancreas of different animal species, the ox and the pig. The substance cannot be obtained in very great amount from the pancreas of either species, and the methods in use for its isolation are far from what might be desired; so that a slight difference observed would be without significance. But we obtained about three and one-half times as much guanylic acid from ox pancreas as from an equal weight of pig pancreas.

All things considered, we are inclined to believe that the guanylic acid of animal tissues has no direct relation to animal nucleic acid, but owes its origin entirely to the nucleic acid of plant food.

<sup>6</sup>Levene and Jacobs: *loc. cit.*





## STUDIES ON FASTING FLOUNDERS.<sup>1</sup>

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In a series of investigations on the nutrition of aquatic animals Pütter<sup>2</sup> propounded the theory that the sea contains considerable quantities of organic matter in solution serving as nutrients for these organisms. The energy required by the aquatic animals is, according to this view, derived from two sources: from the food which is ingested in the ordinary manner, and from the dissolved organic matter which, in the case of fishes, is supposed to be absorbed through the gills.

Simple as this theory may seem in its fundamental proposition, the arguments evolved by Pütter in the development of his thesis rest upon an intricate network of assumptions and offhand calculations. That the gills might act as an organ of absorption is in accordance with their embryological history; but, as Moore<sup>3</sup> points out, we know of no substance in the gills which, by virtue of its strong affinity for the organic material in solution, would remove this material from very large volumes of water. But, whatever may be surmised regarding the gills, Pütter's contention, as far as the experimental proof is concerned, has been shattered at every point.

Pütter's claim that sea water contains large quantities of carbonaceous material in solution has been shown by Henze<sup>4</sup> to be

<sup>1</sup> Published by permission of the Commissioner of Fisheries.

<sup>2</sup> A. Pütter: Die Ernährung der Fische, *Ztschr. f. allg. Physiol.*, ix, p. 147, 1909.

<sup>3</sup> B. Moore, E. S. Edie, E. Whitley, and W. J. Dakin: The nutrition and metabolism of marine animals in relationship to (a) dissolved organic matter and (b) particulate organic matter of sea water, *Biochem. Jour.*, vi, pp. 255-296, 1912.

<sup>4</sup> M. Henze: Bemerkungen zu den Anschauungen Pütters über den Gehalt des Meeres an gelösten organischen Kohlenstoffverbindungen und deren Bedeutung für den Stoffhaushalt des Meeres, *Arch. f. d. ges. Physiol.*, cxxiii, p. 487, 1908.

based on a faulty technique in working with the Messinger wet combustion method. The quantities which Henze's careful chemical analyses of the sea water showed were well within the limits of experimental error. More recently Moore<sup>5</sup> showed that also in the application of the permanganate method for the estimation of organic material Pütter committed serious analytical errors, having mistaken the reduction caused by an excess of chlorides for an indication of an abundance of organic matter. Besides, Moore worked with thoroughly filtered sea water, whereas Pütter seems to have entirely overlooked the importance of this preliminary requisition. The results which Moore found agree absolutely with Henze's, both investigators having shown that the organic matter in the sea water is in practically negligible amounts.

In upholding his thesis Pütter resorted to another line of reasoning, to prove his point by inference. He analyzed the classical case of the Rhine salmon, which, as Miescher has shown, subsists wholly on its own body reserves during the several months which it spends in fresh water. The energy which the salmon should expend, according to Pütter's calculations, in swimming up-stream could not be derived from the oxidation of the material which the salmon loses in the course of the few months of fasting. Hence the conclusion follows that another source of energy must exist, which is the dissolved organic matter absorbed through the gills. This inference would claim some consideration if the energy requirement of the salmon had actually been determined; but, as a matter of fact, Pütter estimates it from some experiments he performed with flounders; in other words, he bases it on another inference. Furthermore, as will be shown, the method employed in studying the energy output of flounders was such as to yield high values.

By far the most valuable evidence which Pütter claims in corroboration of his views has been obtained from a series of experiments in which the oxygen consumption of several kinds of fish has been determined during complete fasting. A study of the

<sup>5</sup> B. Moore, E. S. Edie, and E. Whitley: The nutrition and metabolism of marine animals; the rate of oxidation and output of carbon dioxide in marine animals in relation to the available supply of food in sea water, Report for 1913 on the Lancashire sea-fisheries, xxii, pp. 297-320, Liverpool, 1913.

chemical composition of these fish at the close of the fast enabled him, by comparing it with the initial composition, to find out the loss in each constituent. From this it was a simple matter to calculate the amount of oxygen necessary to burn completely the lost material. The oxygen consumption of the fish has been found to be invariably greater than would be necessary for the combustion of this material, and this result again was considered by Pütter as positive proof that other substances, besides those lost by the animal, had partaken in the metabolism. Though this reasoning seems logical, the argument fails to convince because the total oxygen consumption of the fasting fish has not been actually measured. Pütter performed daily an experiment lasting thirty to sixty minutes; and from the results thus obtained he calculated how much oxygen the fish required for twenty-four hours. Any one familiar with respiration work cannot fail to appreciate that this procedure could only misrepresent the facts. When first put into the jar the fish on which the experiment is to be performed are generally restless for some time, and this is sufficient to increase their respiratory exchange frequently by 50 per cent. In an experiment of long duration this temporary excitement of the fish has only a slight effect on the final results, but in experiments of only thirty minutes or one hour very high values can be found. Pütter's contention that the oxygen consumption of his fish for the entire fasting period was more than that necessary to oxidize the body material is evidently based on an error which has been regularly committed in the experiments and has been augmented through multiplication by a large factor. Indeed, Lipschütz<sup>6</sup> showed that the longer the daily experiments were made, the lower was the calculated amount of oxygen consumed in twenty-four hours.

It seemed to me worth while, in order to settle this question definitely, to undertake a study of the oxygen consumption of fasting fish, avoiding as far as practicable all computations. With this in mind I performed experiments with small flounders whose oxygen consumption was measured continuously throughout the fasting period, except for the few minutes which were lost each time while changing the water of the jars. The two experiments reported in this paper lasted each 671 hours, and the oxygen in-

<sup>6</sup> A. Lipschütz: Über den Hungerstoffwechsel der Fische, *Ztschr. f. allg. Physiol.*, xii, pp. 118-124, 1910.

take was determined for almost 669 hours. In the course of four weeks, therefore, the oxygen consumption for two hours *only* was not directly measured but calculated.

At the end of the experiment both flounders were analyzed and the water, nitrogen, and fat contents determined. Their initial composition, at the beginning of the experiment, was figured out with the aid of the average percentage composition worked out for similar flounders. Owing to the smallness of the sample the inorganic content of the material from the fasting flounders was not determined.

#### METHODS.<sup>7</sup>

The oxygen consumption of the flounders has been determined by the difference in the oxygen content of the water at the beginning and end of an experiment. The sea water was allowed to run into the jars, which had a capacity of about 4½ liters, through a tube reaching down to the bottom. A sample of the water from the jar was siphoned into a small bottle of known volume. The stream through the siphon was maintained continuously for about five minutes, so that the contents of the sample bottle were changed at least twenty times. The jar was closed hermetically by a vaselined plate. At the end of the experiment the plate was removed and a new sample of the water taken with the siphon which extended to about the middle of the jar. The water was drawn about two minutes, the contents of the sample bottle being thus changed about five times. The water was analyzed for oxygen according to Winkler's well known method. All analyses have been made in duplicate, and the results have agreed closely. Since the exact volume of the sample and jar, and the proportion of oxygen dissolved in the sea water at the beginning and end are known, the oxygen consumption for a definite period is easily calculated. A special correction has been made on the basis of blank experiments.

The analyses of the flounders were made in the ordinary way. The animals were washed in distilled water and weighed as soon as all the superficial water was removed. They were desiccated in the open air, then ground up finely, and dried to constant weight. The material was extracted with ether, the residue being weighed as fat. Part of the material after extraction was used for nitrogen determinations by the Kjeldahl method, and the rest was incinerated to find the ash content.

The tables contain a complete record of the results of these experiments. It will be seen that the first flounder, *D*, weighing 5.29 grams at the beginning of the experiment, *i.e.*, twenty-four

<sup>7</sup> The methods employed have already been given in detail. S. Morgulis: Contributions to the gaseous metabolism of fishes, *Proc. of the Biochem. Assoc., Biochem. Bull.*, 1915 (in press).

hours after the last feeding, consumed 0.192 cc. of oxygen per gram per hour, or 0.026 cc. per square centimeter per hour. This animal was very restless at first, but afterwards, and especially towards the end of the experiment, it kept very quiet. The weight was ascertained every four days. The total loss was over one-third of the original weight (34.4 per cent), when I was obliged to sacrifice the animal. In that time (668 hours, 40 minutes) it used up 485.56

TABLE I.

DATE	WEIGHT	SURFACE	EXPERIMENT			TEMPERATURE	OXYGEN CONSUMED			REMARKS
			Be- gin- ning	End	Duration		Total	Per hour per gm.	Per hour per sq.cm.	
July	gm.	sq.cm.	p.m.	a.m.		°C.	cc.	cc.	cc.	
13-14	5.29	38.82	12:45	9:55	21h 10'	20.5	21.45	0.192	0.026	Last feeding July 12.
			a.m.							Somewhat restless.
14-15			10:00	8:55	22h 55'	20.5	23.05			Restless.
15-16			9:00	8:00	23h	20.5	22.48			Restless.
16-17			8:05	9:40	25h 35'	20.0	26.46			Very restless. Lost 10.1 per cent.
17-18	4.76		9:50	8:50	23h	21.0	21.62	0.197		Quiet.
18-19			8:55	10:25	25h 25'	20.5	34.71			Very restless. Swims about in wide circles.
19-20			10:30	10:20	23h 45'	21.0	27.69			Swims about restlessly.
20-21			10:25	9:20	22h 55'	21.5	15.52			Rather quiet. Lost 14 per cent.
21-22	4.55		9:35	9:00	23h 25'	21.5	20.40	0.191		Quiet.
22-23			9:05	8:25	23h 40'	21.0	14.62			Somewhat restless.
23-24			8:30	8:55	24h 25'	21.0	19.04			Swam about restlessly in the beginning.
24-25			9:00	8:20	23h 20'	21.0	17.54			Lost 19.1 per cent.
25-26	4.28		8:30	8:45	24h 15'	21.0	17.67	0.170		Swam about much during afternoon.
26-27			8:50	8:50	24h	20.5	14.55			Quiet.
27-28			8:55	8:55	24h	20.5	16.16			Seems rather quiet.
28-29			9:00	9:15	24h 15'	20.5	13.77			Lost 21 per cent.
29-30	4.14		9:25	9:55	24h 30'	20.5	17.14	0.170		
30-31			10:00	9:15	23h 45'	20.0	17.61			
31-1			9:20	9:55	24h 35'	20.5	17.28			
August			10:00	10:15	24h 15'	21.0	16.47			
1-2			10:20	10:20	24h	21.5	15.32			Lost 27.6 per cent.
3-4	3.83		10:25	10:30	24h 5'	21.0	15.82	0.172		
4-5			10:35	10:35	24h	21.5	13.65			
5-6			10:40	10:40	24h	21.0	10.29			
6-7	3.65		10:45	10:15	23h 30'	21.0	10.84	0.126		Lost 31 per cent.
7-8			10:20	10:50	24h 30'	19.7	10.28			Moves about very little.
8-9			10:55	11:40	24h 45'	17.5	6.89			Very quiet.
9-10			11:45	11:45	24h	17.5	7.24	0.091	0.008	Very quiet.
10	3.47	38.25			668h 40'		485.56			

## Studies on Fasting Flounders

TABLE 11.

DATE	WEIGHT	SURFACE	EXPERIMENT			TEMPERATURE	OXYGEN CONSUMED			REMARKS
			Be- gin- ning	End	Duration		Total	Per hour per gm.	Per hour per sq.cm.	
July	gm.	sq.cm.	p.m.	a.m.		°C.	cc.	cc.	cc.	
13-14	4.04	34.76	12:55 a.m.	10:05	21h 10'	20.5	15.51	0.181	0.021	Last feeding July 13.
14-15			10:10	9:05	22h 55'	20.5	16.54			Animal is very quiet.
15-16			9:10	8:10	23h	20.5	10.33			Animal is very quiet.
16-17			8:15	9:55	25h 40'	20.0	13.12			Lost 7.2 per cent.
17-18	3.75		10:05	9:05	23h	21.0	16.79	0.195		Restless.
18-19			9:10	10:15	25h 5'	20.5	17.16			Somewhat restless.
										Swims about con- siderably.
19-20			10:20	10:30	24h 10'	21.0	15.77			Swims around rest- lessly. Feces.
20-21			10:35	9:40	23h 5'	21.5	15.43			Somewhat restless.
										Feces. Lost 9.4 per cent.
21-22	3.66		9:50	9:10	23h 20'	21.5	17.23	0.201		Very restless. Feces.
22-23			9:15	8:35	23h 40'	21.0	14.08			Swims around rest- lessly.
23-24			8:40	9:05	24h 25'	21.0	17.95			Restless.
24-25			9:10	8:35	23h 25'	21.0	15.16			During early part of experiment swam around much. Lost 16.83 per cent.
25-26	3.36		8:45	8:55	24h 10'	21.0	16.46	0.203		Same.
26-27			9:00	9:00	24h	20.5	13.37			Swam around all after- noon.
27-28			9:05	9:05	24h	20.5	12.26			Restless.
28-29			9:10	9:30	24h 20'	20.5	11.57			Lost 20.3 per cent.
29-30	3.22		9:40	10:05	24h 25'	20.5	14.00	0.178		Restless.
30-31			10:05	9:35	23h 30'	20.0	15.07			Restless.
31-1			9:40	10:05	24h 25'	20.5	16.43			Restless.
August										
1-2			10:10	10:25	24h 15'	21.0	15.66			Very restless.
2-3			10:30	10:30	24h	21.5	13.44			Lost 27 per cent.
3-4	2.95		10:35	10:40	24h 5'	21.0	11.82	0.167		
4-5			10:45	10:45	24h	21.5	11.09			Swims about rest- lessly.
5-6			10:50	10:50	24h	21.0	12.80			Restless. Lost 29.7 per cent.
6-7	2.84		11:00	10:30	23h 30'	21.0	14.72	0.220		Restless.
7-8			10:35	11:05	24h 30'	19.7	12.18			Restless.
8-9			11:10	11:50	24h 40'	17.5	9.90			Very restless towards evening. Quiet next morning.
9-10			11:55	11:55	24h	17.5	8.94	0.139	0.011	Rather quiet. Lost 33.4 per cent.
10	2.69	34.25			668h 45'		394.68			

cc. of oxygen, or an average of about 0.74 cc. per hour. While at the start it consumed 1.02 cc. of oxygen per hour, it took only 0.30 cc. per hour on the last day, or less than one-third. It must be pointed out, however, that at first this flounder was more or less restless, and during the last two to three days, owing to an abrupt change of temperature, its oxygen consumption was greatly reduced.

The second flounder, *E*, was smaller, weighing only 4.04 grams, and fasted likewise 668 hours, 45 minutes. Its oxygen requirement during the second twenty-four hours after the last feeding was 0.181 cc. per gram per hour and 0.021 cc. per square centimeter per hour. This animal behaved very quietly for the first few days, but from the fifth day of the experiment it grew continually more restless. It is interesting to record that though the animal had no food it continued to throw off small quantities of feces until the eighth day. It had lost a third of its body-weight (33.4 per cent) when it was killed at the end of twenty-eight days. It had used up 394.68 cc. of oxygen, or on an average 0.591 cc. per hour. Its oxygen consumption diminished to one-half, being 0.74 cc. and 0.39 cc. per hour on the first and last days of the experiment, respectively.

From Tables III, IV, and V it will be seen that the first flounder, *D*, lost 1.355 grams of water, 0.353 of a gram of protein, and 0.089 of a gram of fat in the course of twenty-eight days of fasting. In other words, almost one-third of the water content of the body had been lost, and about one-half of the protein, while, at the same time, nearly nine-tenths of the fat had been wasted. When the share of each of these constituents in the total loss is considered, it is seen that practically three-fourths of it is water (74.45 per cent), while the protein and fat contribute 18.3 and 4.89 per cent, respectively.

Similarly, flounder *E* had lost 0.992 of a gram of water, 0.300 of a gram of protein, and 0.058 of a gram of fat. In the case of this specimen, however, more of the protein but less of the fat material had been lost. But in both flounders the relative contribution of each of these materials to the total loss is about the same.

With these data it is possible to compute with considerable accuracy the amount of oxygen necessary to oxidize completely the material lost during the fast. Under the heading "Oxygen



required" in the last two tables it will be found that for flounder *D* 520.7 cc. of oxygen would have been necessary, and for flounder *E* 406.9 cc., to burn the material utilized in their maintenance metabolism. These computed figures compare very favorably with the data of the oxygen consumption actually measured and recorded

TABLE III.  
*Composition of 100 grams of flounder.*

	WATER	PROTEIN	FAT	ASH	UNDETERMINED
Normal flounder.....	80.25	13.92	1.89	3.38	0.64
Flounder <i>D</i> , fasting 28 dys.....	83.29	10.41	0.33	?	?
Flounder <i>E</i> , fasting 28 dys.....	83.04	9.11	0.66	?	?

TABLE IV.  
*Composition of flounder D.*

COMPOSITION	TOTAL WEIGHT	WATER	PROTEIN	FAT	ASH	UNDETERMINED
Initial (computed)...	5.29 gm.	4.245 gm.	0.736 gm.	0.100 gm.	0.174 gm.	0.034 gm.
Final (determined)...	3.47 gm.	2.890 gm.	0.383 gm.	0.011 gm.	?	?
Loss in gm.....	1.82 gm.	1.355 gm.	0.353 gm.	0.089 gm.	?	?
Loss in per cent.....	34.40%	31.21%	47.96%	89%		
Relative loss in per cent.....	34.40%	74.45%	18.3%	4.89%		
Oxygen required.....	520.7 cc.		341.0 cc.	179.7 cc.		
Carbon dioxide.....	393.9 cc.		286.3 cc.	127.6 cc.		

TABLE V.  
*Composition of flounder E.*

COMPOSITION	TOTAL WEIGHT	WATER	PROTEIN	FAT	ASH	UNDETERMINED
Initial (computed)...	4.04 gm.	3.242 gm.	0.562 gm.	0.076 gm.	0.133 gm.	0.026 gm.
Final (determined)...	2.69 gm.	2.250 gm.	0.262 gm.	0.018 gm.	?	?
Loss in gm.....	1.35 gm.	0.992 gm.	0.300 gm.	0.058 gm.	?	?
Loss in per cent.....	33.40%	30.60%	53.38%	76.31%		
Relative loss in per cent.....	33.40%	73.49%	22.22%	4.30%		
Oxygen required.....	406.9 cc.		289.8 cc.	117.1 cc.		
Carbon dioxide.....	309.5 cc.		226.35 cc.	83.15 cc.		

in the first two tables. There we found that flounder *D* consumed 485.6 cc. of oxygen, and flounder *E* used up 394.7 cc., or 6.7 and 3 per cent less than was expected from the amount of wasted tissue. It is very significant, and this is a point worth emphasizing, that in every case I find the theoretical oxygen re-

quirement somewhat *higher* than the actually determined amount; while Pütter in his experiments found the reverse. But Pütter did not accurately determine the oxygen consumption of the fasting fish. His method, as was shown above, could not but exaggerate the errors. The difference which I found between the amount of oxygen used up in a respiration experiment lasting practically the full length of the fast, and the oxygen as computed from the wasted body material (6.7 and 3 per cent) is within the limits of experimental error, considering that in the estimation of the initial composition of the flounders, at the moment the fast began, slight errors are unavoidable.

These experiments demonstrate conclusively that aquatic animals depend on particulate food, and subsist on their own tissues when deprived of food. Organic matter dissolved in sea water apparently plays no rôle in the nutrition of these animals. In this connection we may recall that Kerb<sup>8</sup> has shown that aquatic animals do not absorb the sugar in solutions in which they live. The results here retorded justify the statement that the last semblance of evidence in favor of Pütter's hypothesis is disproved.

An examination of the composition of the starved flounders shows that the absolute loss of protein is much greater than the loss of fat; being 4:1 and 5:1 in flounders *D* and *E*, respectively. This fact is very interesting, because in the case of fasting mammals the relation is entirely reversed. Thus in the two famous subjects of fasting experiments, Cetti and Breithaupt, the relation between the consumed protein and fat was, in round numbers, 1:5. In very few instances on record has the protein contributed much more to the total loss of material. The predominant part which protein plays in the fasting metabolism of fish indicates the importance of the nitrogen metabolism of fish as the principal source of energy. The total energy expenditure of flounder *D* was 2399 small calories, and that of the smaller fish, *E*, 1871 calories. Of this amount of energy 64.6 and 61 per cent were derived from protein.

In the last row of Tables IV and V, besides the oxygen required to oxidize completely the body materials, the quantities of carbon dioxide developed in that process are likewise com-

<sup>8</sup> W. Kerb: Über den Nährwert der im Wasser gelösten Stoffe, *Internat. Rev. d. ges. Hydrobiol. u. Hydrogr.*, iii, 1910.

puted. Dividing these figures by the corresponding amount of oxygen, we find that the average respiratory quotient for the whole fasting period is 0.756 and 0.761 for flounders *D* and *E*, respectively, ( $\frac{393.9}{520.7}$  and  $\frac{309.5}{406.9}$ ). These quotients are rather high, but they likewise point to the important contribution of the proteins of the body to the metabolic exchange.

# AN EXAMINATION OF THE FOLIN-FARMER METHOD FOR THE COLORIMETRIC ESTIMATION OF NITROGEN.

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## INTRODUCTORY.

Many micro methods of analysis have recently been described in the literature, which provide for the determination of a great variety of substances. These methods open new avenues of approach to the study of many problems; but for the most part they have been advocated by their authors as not only suitable when only small amounts of material are available, but are offered as complete substitutes of our standard methods of analysis.

In order that any method may reach its full sphere of usefulness it is necessary that its limitations as well as its advantages should be clearly understood. We have had occasion in this laboratory to examine in some detail several of the micro procedures recently advocated and shall report upon some of these methods in subsequent communications. The present paper summarizes our findings concerning the colorimetric method for total nitrogen, as proposed by Folin and Farmer.<sup>1</sup>

### *The known sources of error in the Folin-Farmer colorimetric method.*

The Folin-Farmer method has been advocated by its authors as fully replacing the ordinary Kjeldahl method. The accuracy of the Kjeldahl method is so well known and the amount of work and conclusions based upon its use are so vast, that any method offered as an alternative to it should be subjected to the closest scrutiny. A far closer examination is needed than if the method

<sup>1</sup> O. Folin and C. J. Farmer: this *Journal*, xi, p. 493, 1912.

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were simply offered as available when one *must* have results at once, or when only a small amount of material is available.

The colorimeter is at the basis of the method we are discussing and at the outset it is well to remember that the accuracy of the best colorimeter available—the DuBoscq—is limited to about 1 per cent of the quantity of substance employed for the determination.

A possible second 1 per cent error is introduced in the measurement of 1 cc. of solution employed for a determination. One cc. pipettes<sup>2</sup> are not accurate to within less than 1 per cent when most carefully used; under ordinary conditions the error is apt to be much larger.

At the outset, then, we meet two possible 1 per cent errors, which the best technique cannot guard against. Either one or both of these errors may be apparent in any one determination, and may or may not neutralize the other. When it is remembered that the Kjeldahl method, as it is ordinarily employed, with 25 to 50 mgm. of nitrogen for each determination, has an accuracy of 0.1 to 0.2 per cent when properly carried out, it will be seen that the Folin-Farmer method unquestionably has limitations in the direction of accuracy which do not apply to the Kjeldahl process.

A further point which we feel has not been sufficiently emphasized by Folin and Farmer and which is even of more importance in connection with the work upon blood of Folin and Denis<sup>3</sup> is the question of purity of reagents as regards the freedom from ammonium salts. When working with Nessler's reagent it is well to bear in mind that most substances will give a fair test for ammonia with this solution. Furthermore, the quantities of ammonia present in some instances are quite sufficient to represent 1 to 3 per cent of the 1 mgm. which is used in a determination in urine. We have found that ordinary c.p. potassium sulphate contains from 0.005 to 0.01 mgm. of nitrogen per gram, from which it can be freed only by recrystallizing twice from pure distilled water.

Folin and collaborators have nowhere mentioned that it is impossible to obtain sulphuric acid free from ammonia. Ordinarily

<sup>2</sup> Folin and Farmer advocate the use of the Ostwald pipette. We have obtained better results using the Mohr pipette, graduated in  $\frac{1}{100}$  cc.

<sup>3</sup> O. Folin and W. Denis: this *Journal*, xi, p. 527, 1912.

the c.p. acid contains from 0.01 to 0.03 mgm. of ammonia per cubic centimeter, a quantity equivalent to from 1 to 3 per cent of the quantity of nitrogen taken for a urine analysis in this method.

It may be replied that the lack of purity of reagents is not a drawback, since a blank can be run and a correction made. As a matter of fact, however, results are apt to be less satisfactory where a correction is made for such quantities of ammonia as are indicated above, than where such corrections are omitted. This fact is probably due to two factors: first, that the accuracy of the method is not up to 3 per cent of 1 mgm. of nitrogen; and, second, that other errors partially counterbalance the ammonia present in the reagents.

Perhaps one of the most interesting points we have encountered in connection with the investigation of the Folin-Farmer and the Folin-Denis nitrogen method was in relation to the ammonia in air, and to the question of complete absorption of ammonia from air by acid solutions. Throughout the work we have employed the following technique for the detection and relative estimation of small amounts of ammonia. The procedure was to collect the ammonia in a few cubic centimeters of dilute hydrochloric acid in a test-tube, nesslerize this solution with a measured volume of reagent, and add the same volume of reagent to test-tubes containing an equal volume of solution in which were varying amounts of ammonia, usually 0.01, 0.02, and 0.03 mgm. of nitrogen,<sup>4</sup> and compare the colors obtained.

With such technique it is easy to demonstrate that when ordinary laboratory air passes rapidly through a single wash-bottle of dilute or 50 per cent sulphuric acid for ten minutes, from 0.01 to 0.04 mgm. of ammonia will escape absorption by the acid. This is even more true of the dilute acid used by Folin in the absorption of the ammonia to be determined. The contents of a test-tube arranged so that the air passes from the Folin absorption flask through 2 cc. of water containing 1 cc. of  $\frac{N}{10}$  hydrochloric acid will show that a few hundredths of a milligram of nitrogen have escaped absorption. When it is remembered that 0.01 mgm. represents 1 per cent of the quantity of nitrogen used in a determination, this fact is of some importance. It is of far more interest in connection

<sup>4</sup> These quantities of ammonia are readily obtained by dilution of the standard solution.

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with blood work of Folin and Denis,<sup>5</sup> where the total quantity of nitrogen estimated frequently did not exceed a few hundredths of a milligram.

A further point in this same connection is the fact that if *ammonia-free* air (obtained by passing the air through three tall wash-bottles containing varying concentrations of sulphuric acid) is passed rapidly for ten minutes through 1 cc. of  $\frac{N}{16}$  hydrochloric acid and 2 cc. of water, both ammonia-free, contained in an open test-tube (or even if the test-tube has a small funnel in the neck), the solution at the end of the aeration will, upon being nesslerized, be found to contain from 0.005 to 0.02 mgm. of ammonia nitrogen. In this instance the acid solution absorbs ammonia from the air of the room due to the spattering during aeration. If the air is led into the test-tube through a tube contained in a two-holed stopper, with another tube for an outlet, the absorption of ammonia from the air in the room is nearly altogether prevented.

These findings have led us to question the accuracy of the results reported by Folin and Denis<sup>6</sup> in connection with ammonia in blood. These writers report figures for ammonia in various bloods out to an almost infinitely small amount (0.05 mgm. per 100 cc. of blood), yet they report no special precautions to guard against the sources of error mentioned above.

### *Results of urine analysis by the colorimetric method as compared with the Kjeldahl method.*

In Table I will be found reported analyses of some seventy samples of urine, both by the Folin-Farmer method and by the Kjeldahl process. As regards the technique employed, we may say that no pains were spared in the carrying out of either procedure to obtain the best possible results. The glassware employed was all standardized, and the standard acid and alkali for the Kjeldahl method were corrected with the greatest exactness. As a standard solution in the Folin-Farmer method, we compared and frequently used three different solutions. One was prepared from ammonium sulphate, obtained as described by Folin,<sup>7</sup> one

<sup>5</sup> Folin and Denis: this *Journal*, xi, p. 161, 1912.

<sup>6</sup> Folin and Denis: this *Journal*, xi, p. 161, 1912.

<sup>7</sup> One sample we prepared ourselves; a second was obtained from Dr. Emerson of Boston; both had the same value.

from Kahlbaum's ammonium chloride, "zur Analyse," while a third was prepared from the urines to be analyzed.<sup>8</sup> In this latter case a measured volume of a sample of urine which had previously been analyzed by Kjeldahl's method was digested and then distilled into the theoretical quantity of standard acid, and the distillate diluted to a definite volume.

All of these standards had the same color value when nesslerized.

The air current was obtained from a Crowell pressure blower.<sup>9</sup> The air current was slow during the first two minutes, and as rapid as the apparatus would stand for the last eight minutes.

A comparison of the results obtained by the Folin-Farmer method with those obtained by the Kjeldahl will show that there are discrepancies between the two methods of from -11 per cent to +3 per cent. The large majority of the results range from 2 to 4 per cent lower by the Folin-Farmer method than by the Kjeldahl procedure.

An interesting point in connection with the Folin-Farmer procedure is that although one is using seemingly exactly the same technique, many determinations will show a difference from the Kjeldahl method of only about 2 per cent, while others will show a variation of 8 to 10 per cent.<sup>10</sup>

In view of these results it would appear that the Folin-Farmer method should not be employed in urine analysis where certain

<sup>8</sup> We have not experienced the difficulties reported by Folin and Farmer as regards the obtaining of pure ammonium salts on the market. Kahlbaum's "zur Analyse" ammonium chloride is altogether free from pyridine bases or other impurities, at least in detectable quantities.

<sup>9</sup> We believe that if the air current is to be employed for the transfer of the ammonia, a positive pressure blower is essential in most laboratories. We have found numerous laboratories (our own included) where the water pressure is not nearly equal to the forty pounds required to operate a suction pump satisfactorily for this method. Even where the water pressure may be adequate at times errors are apt to occur due to a change in such pressure without notice. Compare also F. C. McLean and L. Selling: this *Journal*, xix, p. 36, 1914.

<sup>10</sup> We believe that the large error not infrequently encountered when using the Folin-Farmer procedure may be due to the fact that while in many determinations the errors, inherent in the method, may neutralize each other, sometimes they may all tend in the same direction, thus producing a result which is incorrect by from 5 to 10 per cent.



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and accurate results are desired. We should, for example, never think of using the Folin-Farmer method in making a nitrogen balance on an animal. A variation of a gram of nitrogen per day by the two methods is not at all uncommon when working on human urine.

### *Certain modifications of the Folin-Farmer method.*

During the work on the Folin-Farmer method two modifications of the procedure were developed and the results obtained with them are recorded in columns three and four of Table I. In Modification No. 1 we have employed distillation by boiling instead of by the air current. This procedure is simpler and more rapid than where the air current is employed and eliminates the uncertainties arising from an insufficient air current and from failure of complete absorption. The digestion is carried out in a test-tube exactly as described by Folin and Farmer, two small glass beads being used to prevent bumping. After the mixture has partially cooled, 7 cc. of water are added and the tube is closed by inserting a two-holed rubber stopper, through one hole of which passes a long tube, reaching nearly to the bottom of the test-tube, and roughly standardized to hold 3 cc., while through the second hole passes a short tube, bent for connecting to a condenser (see Figure 1).

The long tube should be previously filled with 3 cc. of saturated sodium hydroxide solution by suction and closed by means of a rubber tip and a pinch-cock. The outlet tube is then connected with the condenser.<sup>11</sup> A volumetric flask containing 1 to 2 cc. of  $\frac{N}{10}$  hydrochloric acid and enough water to cover the connecting tube is used as a receiver. The alkali is allowed to run into the test-tube, and the fluids are mixed by blowing a few

<sup>11</sup> The complete apparatus is shown in Figure 1. A small Liebig condenser which may be easily prepared in the laboratory suffices. A piece of glass tubing about 30 mm. by 150 mm. is fitted on each end with a double-holed cork or rubber stopper. One long glass tube of about 4-6 mm. inside diameter goes through these stoppers and serves as a condensing tube. Two short bent glass tubes serve as inlet and outlet for the cooling water. The lower end of the condenser is connected with a glass tube (or, better, an old pipette, to prevent back suction) which reaches into the volumetric flask.

TABLE 1.\*

URINE SAMPLE NO.	RESULT OF ANALYSIS BY KJELDAHL METHOD	RESULT OF ANALYSIS BY FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 1 OF FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 2 OF FOLIN-FARMER METHOD
		Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method
1	4.165	3.720 -10.67	4.020 -3.47	4.020 -3.47
2	3.372	3.101 -8.04	3.323 -1.44	3.311 -1.81
3	3.590		3.715 +3.48	3.570 -0.56
4	3.850	3.715 -3.51	3.845 -0.13	3.788 -1.61
5	3.414	3.289 -3.67	3.355 -1.73	3.495 +2.38
6	3.337	3.470 +0.99	3.423 +2.57	3.311 -0.78
7	3.313	3.165 -4.48	3.423 +3.33	3.350 +1.12
8	3.672	3.845 +4.72	3.756 +2.28	3.648 -0.65
9	7.660	7.580 -1.05	7.873 +2.74	7.220 -5.74
10	3.260	3.225 -1.07	3.289 +0.88	3.268 +0.25
11	3.386	3.289 -2.86	3.571 +5.46	3.703 +9.35
12	3.456	3.333 -3.55	3.571 +3.32	3.571 +3.32
13	3.506	3.448 -1.65	3.333 -4.93	3.571 +1.85
14	3.400	3.423 +0.67	3.448 +1.41	3.350 -1.47
15	3.330	3.423 +2.79	3.378 +1.44	3.225 -3.15
16	3.414	3.225 -5.53	3.423 +0.26	3.448 +0.99
17	3.225	3.125 -3.23	3.205 -0.61	3.225 =0
18	3.130	3.125 -0.16	3.205 +2.34	3.298 +5.04
19	3.310	3.227 -2.50	3.355 +1.39	3.333 +0.69

\* The figures represent grams of nitrogen in 24 hour samples of urine.

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TABLE I—Continued.

URINE SAMPLE NO.	RESULT OF ANALYSIS BY KJELDAHL METHOD	RESULT OF ANALYSIS BY FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 1 OF FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 2 OF FOLIN-FARMER METHOD
		Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method
20	3.310	3.125 -5.59	3.311 +0.03	3.214 -2.90
21	3.287	3.185 -3.10	3.325 +1.15	3.325 +1.15
22	3.287		3.311 +0.72	3.288 +0.03
23	3.412	3.325 -2.55	3.497 +2.49	3.423 +0.32
24	3.412	3.325 -2.55	3.401 -0.32	3.268 -4.22
25	2.770	2.787 +0.61	2.778 +0.28	2.621 -5.38
26	2.770	2.703 -2.42	2.747 -1.08	2.663 -3.86
27	3.084	3.227 +4.63	3.227 +4.63	3.011 -2.37
28	2.670	2.778 +4.04	2.825 +5.80	2.604 -2.46
29	2.670	2.710 +1.49	2.797 +4.75	2.591 -2.96
30	2.888	2.747 -4.87	2.959 +2.45	2.874 -0.48
31	2.888		2.941 +1.83	2.924 +1.24
32	3.260	2.959 -9.24		3.325 +1.99
33	3.260	3.086 -5.31	3.288 +0.85	3.185 -2.30
34	3.238	3.086 -4.69	3.145 -2.87	2.770 -14.20
35	3.238	3.125 -3.48	3.227 -0.34	2.747 -15.15
36	3.421	3.125 -8.65	3.125 -8.65	3.325 -2.80
37	3.218	3.011 -6.42	3.125 -2.79	3.227 +0.28
38	2.815	2.747 -2.41	2.763 -1.84	2.763 -1.84
39	2.866	2.703 -5.67	2.841 -0.87	2.762 -3.62

TABLE 1—Continued.

URINE SAMPLE NO.	RESULT OF ANALYSIS BY KJELDAHL METHOD	RESULT OF ANALYSIS BY FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 1 OF FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 2 OF FOLIN-FARMER METHOD
		Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method
40	3.395	3.200 -5.73	3.346 -1.44	3.346 -1.44
41	2.530	2.304 -8.93	2.439 -3.58	2.424 -4.18
42	3.413	3.247 -4.86	3.322 -2.66	3.322 -2.66
43	3.141	2.890 -8.00	3.106 -1.11	3.125 -0.50
44	3.358	3.106 -7.50	3.423 +1.93	3.350 -0.23
45	3.323	3.268 -1.62	3.350 +0.81	3.325 +0.06
46	3.246	3.227 -0.57	3.225 -0.63	3.205 -1.24
47	2.340	2.294 -2.39	2.326 -0.59	2.370 +1.28
48	2.620	2.451 -6.44	2.591 -1.10	2.621 +0.04
49	2.522	2.500 -0.88	2.513 -0.36	2.525 +0.11
50	3.871	3.758 -2.92	3.818 -1.37	3.846 -0.64
51	3.905	3.730 -4.47	3.875 -0.76	3.846 -1.51
52	4.404	4.168 -5.97	4.348 -1.27	4.425 +0.47
53	4.384		4.323 -1.39	4.560 +4.01
54	4.447	4.286 -3.62	4.444 -0.07	4.477 +0.68
55	4.278	4.444 +3.87	4.253 -0.58	4.286 +0.20
56	4.404	4.286 -2.68	4.478 +1.68	4.444 +0.91
57	4.278	4.189 -2.07	4.237 -0.95	4.250 -0.65
58	4.130	4.098 -0.77	4.166 +0.87	4.121 -0.22
59	4.110	4.054 -1.36	4.121 +0.26	4.121 +0.26

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TABLE I—Concluded.

URINE SAMPLE NO.	RESULT OF ANALYSIS BY KJELDAHL METHOD	RESULT OF ANALYSIS BY FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 1 OF FOLIN-FARMER METHOD	RESULT OF ANALYSIS BY MODI- FICATION NO. 2 OF FOLIN-FARMER METHOD
		Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method	Per cent of differ- ence from Kjeldahl method
60	4.152	4.166 +0.33	4.180 +0.88	4.166 +0.33
61	3.490	3.330 -4.58	3.600 +3.15	3.240 -7.16
62	3.600	3.270 -9.16	3.510 -2.50	3.690 +2.50
63	3.590	3.330 -4.45	3.63 -1.94	3.750 +4.44
64	3.780	3.510 -7.10	3.780 =0	3.810 +0.78
65	3.860	3.750 -2.85	3.810 -1.29	3.900 +1.03
66	3.770	3.660 -2.92	3.780 +0.26	3.890 +3.18
67	3.690	3.450 -6.50	3.870 +4.87	3.810 +3.25
68	3.030	2.670 -11.88	2.85 -5.94	2.850 -5.94
69	3.120	3.030 -2.88	3.180 +1.92	3.060 -1.92
70	3.170	3.030 -4.41	3.150 -0.02	3.060 -3.47
71	14.94	14.00 -6.29	14.86 -0.53	14.740 -1.33

bubbles of air through the apparatus. The test-tube is then heated to vigorous boiling (over a large free flame), the distillation being continued until a separation of salts occurs in the test-tube and the mixture begins to bump. This distillation requires about two minutes. The test-tube is then disconnected from the condenser and the latter washed down with a few cubic centimeters of water. The liquid in the receiving flask is diluted and nesslerized, as in the Folin-Farmer method.

This procedure is more convenient than the air distillation, and an examination of the results by it (Table I) will show that it is also more reliable. Still the results obtained do not at all war-

rant its substitution for the Kjeldahl method in urine analysis. We do, however, believe the technique to be of real service in blood analysis, as of greater rapidity and accuracy than the air distillation and because the volumetric flask used as a receiver may be as small as necessity requires, at least to 10 cc.<sup>12</sup>

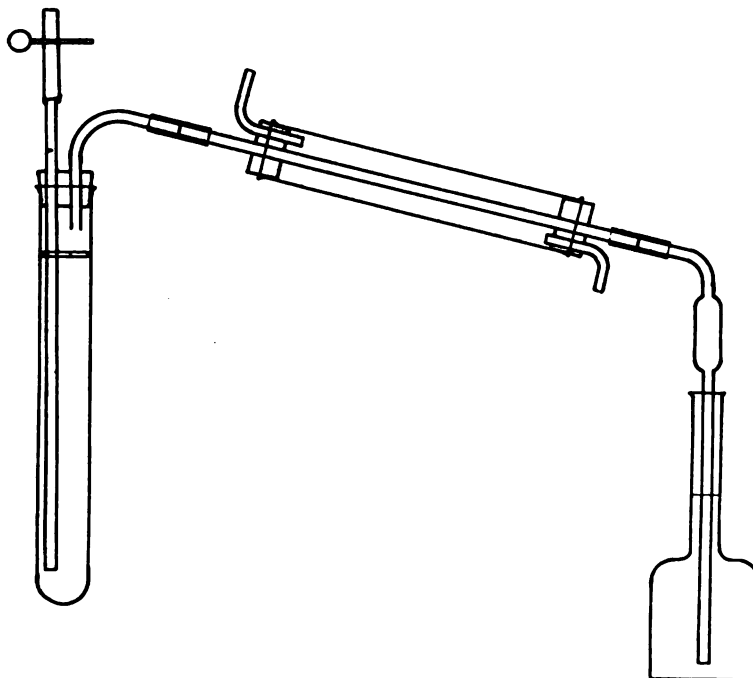


FIG. 1.

In Table I under the column headed Modification No. 2 are recorded analyses made by a direct dilution method, where distillation was wholly dispensed with.<sup>13</sup> The technique employed

<sup>12</sup> In case a 10 cc. flask is used, only 6 cc. of water are added to the digested mixture in the test-tube, and 1 cc. of  $\frac{N}{10}$  hydrochloric acid is used in the receiver. The washing down of the condenser can be dispensed with.

<sup>13</sup> Gulick (this *Journal*, xviii, p. 541, 1914) has very recently proposed a procedure similar to this one. Our analyses were made a year or two ago and we have not had an opportunity to make many trials of the procedure Gulick suggests. He reports analyses of only one sample of urine by his method.

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was as follows: A quantity of urine containing about 5 mgm. of nitrogen (1 cc. of a slightly diluted sample of urine is usually the right amount) is placed in a large test-tube and digested as in the Folin-Farmer method, except that no copper sulphate is employed. After cooling, the mixture is washed quantitatively into a 50 cc. volumetric flask, cooled, and diluted to the mark. Ten cc. (or such a volume as will contain 0.7 to 1.5 mgm. of nitrogen) are transferred to a 100 cc. volumetric flask and diluted and nesslerized as usual. The standard is 5 cc. of a solution containing 20 mgm. of ammonia nitrogen, 4 cc. of sulphuric acid, and 4 gm. of potassium sulphate in 100 cc. of solution. Both the unknown and the standard are nearly neutralized by adding five to six drops of concentrated sodium hydroxide solution before being nesslerized.<sup>14</sup>

The results by this method are more satisfactory than by the original Folin-Farmer procedure, but are scarcely as good as by the distillation method. We feel that the direct method is not quite so safe as the distillation by boiling. The latter method practically never shows such large variations from the Kjeldahl results as are sometimes obtained with the direct dilution method or with the original Folin-Farmer procedure.

### SUMMARY AND CONCLUSIONS.

We may summarize the results reported in this paper as follows:

1. Certain sources of error in the Folin-Farmer method are mentioned.
2. Analysis of some seventy samples of urine by the Folin-Farmer colorimetric method shows that this method usually agrees with the Kjeldahl method within about 2 or 3 per cent. For the most part the figures by the Folin-Farmer method are appreciably lower than by the Kjeldahl process, but variations occur between +4 per cent and -11.8 per cent of the total quantity of nitrogen present.
3. The Folin-Farmer method is not to be regarded as equivalent to the ordinary Kjeldahl procedure in accuracy or reliability.

<sup>14</sup> The quantities of salts present give no trouble due to development of turbidity, but affect the reading somewhat, so that standard and unknown should contain approximately the same quantities.

4. Two modifications of the Folin-Farmer method are presented, one of which is recommended as of service in blood analysis or where only small quantities of material are available.

5. The results reported show that the Folin-Farmer method can be employed with advantage in any instances where the total quantity of nitrogen present is very small (as in the determination of the total non-protein nitrogen of the blood), because a high percentage error is of much less importance in such analyses.





## A METHOD FOR THE ESTIMATION OF SUGAR IN SMALL QUANTITIES OF BLOOD.<sup>1</sup>

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The quantitative determination of blood sugar is growing more and more indispensable, both to the clinician and to the laboratory worker. In the literature we find frequent references to the significance of such determinations. Thus Weiland<sup>2</sup> has pointed out the importance of the blood sugar picture for diagnostic purposes. On the first page of his book on "Glycosuria and Diabetes" Allen<sup>3</sup> says: "There is a generally recognized clinical need of a method for quantitative blood sugar determination which shall be quick, accurate, and adapted for use with small blood samples." The method proposed in the present paper has, we believe, all of these qualifications.

Two distinct processes are involved in a quantitative determination of blood sugar: (1) the removal of blood protein, and (2) the determination of the sugar in the protein-free filtrate. In the first of these operations lies perhaps the chief difficulty and uncertainty in methods of blood sugar analysis, not on account of the difficulty of removing the protein, but because of the danger of carrying down some sugar at the same time. Many of the older methods call for the washing of the precipitated protein

<sup>1</sup> A preliminary report of the method described in this paper was made before the Society for Experimental Biology and Medicine, Dec. 17, 1913 (*Proc. Soc. Exper. Biol. and Med.*, xi, p. 57, 1913-14.)

<sup>2</sup> W. Weiland: *Centralbl. f. d. ges. Physiol. u. Path. d. Stoffw.*, v, p. 481, 1910.

<sup>3</sup> F. M. Allen: *Studies concerning Glycosuria and Diabetes*, Cambridge, 1913.

entirely free from reducing substances, a matter of much difficulty owing to the gelatinous character of the protein mass. In most of the newer methods aliquots of the filtrate obtained after precipitation of the protein are taken for the sugar determination. In such cases it is necessary to show that there is no adsorption of sugar by the protein mass.

In the following brief summary of the literature on blood sugar determination we shall consider the two steps in the process separately. Claude Bernard,<sup>4</sup> who first demonstrated the presence of sugar in normal blood in 1849, used acetic acid and sodium sulphate to remove the protein. Lisbonne<sup>5</sup> has recently revived the use of this method. Seegen<sup>6</sup> employed the Schmidt-Mulheim procedure of protein precipitation with ferric acetate. In the original method of Abeles,<sup>7</sup> as well as in the Bang<sup>8</sup> modification where the washing of the protein coagulum was facilitated by the use of the centrifuge, the protein was removed by the addition of an alcoholic zinc acetate solution. These methods are no longer used. Schenck<sup>9</sup> took advantage of the property of mercuric chloride and hydrochloric acid to precipitate protein; while Lépine and Boulud,<sup>10</sup> Schöndorff,<sup>11</sup> and Bierry and Portier<sup>12</sup> adopted a somewhat similar method proposed by Patein and Dufou; namely, the use of mercuric nitrate. None of the older methods of protein precipitation as a preliminary to blood sugar analysis has received more general usage than that of Waymouth Reid,<sup>13</sup> as recommended by Vosburgh and Richards<sup>14</sup> in this country. Phosphotungstic acid in dilute hydrochloric acid is the protein precipitant in this method. The precipitated protein mass is hard and granular and hence may be ground up in a mortar and readily washed free from reducing substance. Oppler<sup>15</sup> has made use of phosphotungstic acid and neutral lead acetate to remove the protein in making blood sugar estimations. Alcohol alone or in combination with other methods has received considerable favor (Bang and

<sup>4</sup> Claude Bernard: *Mém. de la Soc. biol.*, i, p. 121, 1849.

<sup>5</sup> M. Lisbonne: *Compt. rend. Soc. de biol.*, lxxiv, p. 474, 1913.

<sup>6</sup> J. Seegen: *Centralbl. f. Physiol.*, vi, p. 501, 1892-93.

<sup>7</sup> M. Abeles: *Ztschr. f. physiol. Chem.*, xv, p. 495, 1891.

<sup>8</sup> I. Bang: *Festschrift für Olof Hammarsten*, Upsala, 1906.

<sup>9</sup> F. Schenck: *Arch. f. d. ges. Physiol.*, lv, p. 203, 1894.

<sup>10</sup> P. Lépine and Boulud: *Jour. de physiol. et de path. gén.*, xiii, p. 178, 1911.

<sup>11</sup> B. Schöndorff: *Arch. f. d. ges. Physiol.*, cxxi, p. 572, 1908.

<sup>12</sup> Bierry and Portier: *Compt. rend. Soc. de biol.*, lxvi, p. 577, 1909.

<sup>13</sup> E. Waymouth Reid: *Jour. Physiol.*, xx, p. 316, 1896.

<sup>14</sup> C. H. Vosburgh and A. N. Richards: *Am. Jour. Physiol.*, ix, p. 35, 1903.

<sup>15</sup> B. Oppler: *Ztschr. f. physiol. Chem.*, lxiv, p. 393, 1910.

coworkers,<sup>16</sup> Tachau,<sup>17</sup> etc.). Herzfeld<sup>18</sup> has employed metaphosphoric acid as a blood protein precipitant.

Rona and Michaelis<sup>19</sup> have introduced a method of protein precipitation entirely new to blood sugar determinations; namely, the use of colloids. The principle of this method has been adequately explained by Macleod.<sup>20</sup> Michaelis and Rona<sup>21</sup> recommended the use of kaolin and colloidal iron, showing preference for the latter in papers published soon after their first. In the use of colloids an electrolyte is necessary. Rona and Michaelis<sup>22</sup> employed sodium sulphate and Rochelle salt. For whole human blood Rona and Takahashi<sup>23</sup> have advised the use of monosodium phosphate. The method of Michaelis and Rona has been criticized by Oppler;<sup>24</sup> but Schirokauer,<sup>25</sup> Moekel and Frank,<sup>26</sup> Frank,<sup>27</sup> Wilenko,<sup>28</sup> and many others have used it extensively with admirable success. Without a doubt, colloidal iron as a blood protein precipitant has received more general use in blood sugar analysis than any of the procedures previously mentioned.

After the protein has been removed from the blood, several methods are available for the determination of the sugar in the protein-free filtrate. Fermentation by yeast, although possible of application, is seldom employed. The polariscopic determination is very accurate when a sufficient quantity of blood has been taken, and this method has the support of a large number of investigators. Likewise the reduction of solutions of cupric salts has been widely used. The precipitated cuprous oxide may be determined gravimetrically as such or as cupric oxide, the methods being so well known that a description of them here would be superfluous. In the use of copper solutions the degree of reduction, and hence the quantity of sugar, may be determined titrimetrically. The method of Bertrand,<sup>29</sup> in

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<sup>16</sup> I. Bang: *Der Blutzucker*, Wiesbaden, 1913; Bang: *Biochem. Ztschr.*, vii, p. 327, 1908; I. Bang, H. Lyttkens, and J. Sandgren: *Ztschr. f. physiol. Chem.*, lxxv, p. 497, 1910.

<sup>17</sup> H. Tachau: *Deutsch. Arch. f. klin. Med.*, cii, p. 597, 1911.

<sup>18</sup> E. Herzfeld: *Ztschr. f. physiol. Chem.*, lxxvii, p. 420, 1912.

<sup>19</sup> P. Rona and L. Michaelis: *Biochem. Ztschr.*, vii, p. 329, 1908.

<sup>20</sup> J. J. R. Macleod: *Diabetes: Its Pathological Physiology*, International Medical Monographs, New York, 1913, p. 28.

<sup>21</sup> L. Michaelis and P. Rona: *Biochem. Ztschr.*, viii, p. 356, 1908; xiv, p. 476, 1908.

<sup>22</sup> Rona and Michaelis: *loc. cit.*

<sup>23</sup> P. Rona and D. Takahashi: *Biochem. Ztschr.*, xxx, p. 99, 1911.

<sup>24</sup> B. Oppler: *Ztschr. f. physiol. Chem.*, lxxv, p. 71, 1911.

<sup>25</sup> H. Schirokauer: *Berl. klin. Wchnschr.*, xlix, p. 1783, 1912.

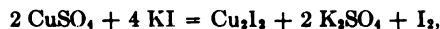
<sup>26</sup> K. Moekel and E. Frank: *Ztschr. f. physiol. Chem.*, lxxv, p. 323, 1910; xlix, p. 85, 1910.

<sup>27</sup> E. Frank: *Ztschr. f. physiol. Chem.*, lxx, p. 129, 1910-11.

<sup>28</sup> G. G. Wilenko: *Arch. f. exper. Path. u. Pharmacol.*, lxviii, p. 297, 1912.

<sup>29</sup> G. Bertrand: *Bull. Soc. chim.*, xxxv, p. 1285, 1906.

which the precipitated cuprous oxide is filtered off, dissolved in a solution of ferric sulphate and sulphuric acid, and titrated with potassium permanganate, has had extensive use. Michaelis<sup>30</sup> has recently modified Bertrand's method so as to make it available for the determination of sugar in small amounts of blood. The reduced cuprous oxide of Fehling's solution is determined by dissolving it in Bertrand's ferric sulphate-sulphuric acid solution and titrating with  $\frac{N}{100}$  potassium permanganate. Several methods have been proposed, the basis of which is the determination of copper left in solution after reduction. Thus Lehmann's<sup>31</sup> method depends on the reaction



the liberated iodine being determined by titration with thiosulphate. In Bang's<sup>32</sup> older titration method the unreduced copper is determined by titration with hydroxylamine. Flatow<sup>33</sup> also uses hydroxylamine in determining unreduced ammoniacal copper in this way. In Bang's<sup>34</sup> newer method, which is microchemical in that only very small quantities of blood are necessary for a determination, the unreduced copper is determined by titration with  $\frac{N}{100}$  or  $\frac{N}{200}$  iodine solution.

The goal for which every investigator working on new blood sugar methods has been striving is to develop a procedure which will require only very small amounts of blood. Bang<sup>34</sup> with his microchemical determination, and Michaelis<sup>30</sup> with his modified Bertrand method have accomplished this; but with these exceptions all the procedures above mentioned require at least 10 cc. and preferably 25 cc. or more of blood for a single determination. Obviously this is a serious disadvantage. The method of Tachau<sup>37</sup> requires 5 to 10 cc. of blood and depends on the reduction of mercuric cyanide (Knapp's solution) in alkaline solution, the excess of mercuric cyanide being determined gravimetrically. Most of the microchemical procedures for blood sugar analysis introduced in the last few years have been based on color reactions. These may be divided into two classes: (1) titrimetric, and (2) colorimetric, meaning a determination with a colorimeter of the intensity of the color reaction. In the method of Herzfeld<sup>38</sup> the protein-free blood filtrate is made alkaline and titrated with methylene blue while boiling, the blood sugar decolorizing the dye. The decolorization of an alkaline safranin solution by dextrose has been used by Hasselbach and

<sup>30</sup> L. Michaelis: *Biochem. Ztschr.*, lix, p. 166, 1914.

<sup>31</sup> K. B. Lehmann: *Arch. f. Hyg.*, xxx, p. 267, 1897.

<sup>32</sup> Bang: *Der Blutzucker*, Wiesbaden, 1913.

<sup>33</sup> Flatow: *Deusch. Arch. f. klin. Med.*, cv, p. 58, 1911-12.

<sup>34</sup> Bang: *Der Blutzucker*, Wiesbaden, 1913.

<sup>35</sup> Bang: *Der Blutzucker*, Wiesbaden, 1913.

<sup>36</sup> Michaelis: *loc. cit.*

<sup>37</sup> Tachau: *loc. cit.*

<sup>38</sup> Herzfeld: *loc. cit.*

Lindhard<sup>39</sup> as the basis of a titrimetric method for the determination of sugar in urine.

Of the colorimetric methods, that of Wacker<sup>40</sup> depends on the reaction of paraphenylhydrazine hydrosulphate and sodium hydroxide with carbohydrates, the amount of color produced being proportional to the blood sugar. Reicher and Stein<sup>41</sup> have applied the Molisch reaction—the production of a red color by carbohydrates with  $\alpha$ -naphthol and sulphuric acid—to the quantitative determination of blood sugar. Forschbach and Severin<sup>42</sup> have modified the Autenreith-Tesdorpf<sup>43</sup> procedure of estimating sugar in urine for the quantitative determination of blood sugar. In this method Bang's solution is reduced by the sugar contained in a small amount of blood, the degree of decolorization being then determined with a colorimeter. According to Allen,<sup>44</sup> all of these colorimetric methods tend to give high results.

Shaffer<sup>45</sup> has recently suggested a procedure for the determination of blood sugar in 5 cc. of blood, in which the blood is freed from protein by coagulation by heat and the use of colloidal iron, the filtrate being boiled with Fehling's solution, and the reduced copper measured colorimetrically, or by titration as in Bertrand's method. Shaffer reports very low values for the sugar content of normal dog's blood by his method, but offers no comparative figures obtained by any standard method upon the same samples of blood, or indeed upon the blood of any species. Since the preliminary work upon the method we wish to report involved some studies practically duplicating Shaffer's procedure, except that they were carried out upon larger volumes of blood, and the results were compared with the figures obtained upon the same samples of blood by the Waymouth Reid gravimetric method, we shall discuss certain phases of Shaffer's method, although we have not tested the method using his exact technique.

Shaffer precipitates the proteins from the blood by heating, addition of acetic acid, and of colloidal iron, and a little sodium sulphate. The filtrate is boiled with Fehling's solution, and the precipitated cuprous oxide is determined. Shaffer himself raises the question as to whether cuprous oxide is completely precipitated from such filtrates, and shows that glucose

<sup>39</sup> K. A. Hasselbach and J. Lindhard: *Biochem. Ztschr.*, xxvii, p. 273, 1910.

<sup>40</sup> L. Wacker: *Ztschr. f. physiol. Chem.*, lxvii, p. 197, 1910.

<sup>41</sup> K. Reicher and E. H. Stein: *Verhandl. d. Cong. f. inn. Med.*, xxvii, p. 401, 1910; *München. med. Wchnschr.*, lvii, p. 1032, 1910; *Biochem. Ztschr.*, xxxvii, p. 321, 1911.

<sup>42</sup> Forschbach and Severin: *Arch. f. exper. Path. u. Pharmacol.*, lxviii, p. 341, 1912.

<sup>43</sup> W. Autenrieth and Th. Tesdorpf: *München. med. Wchnschr.*, lvii, p. 1780, 1910.

<sup>44</sup> Allen: *loc. cit.*

<sup>45</sup> P. A. Shaffer: *Proc. Soc. Biol. Chem.*; this *Journal*, xvii, p. xlii, 1914; xix, p. 285, 1914.

added to ox blood which has stood about the laboratory for a few days and has lost its reducing power, is not quantitatively recovered; i.e., some of the cuprous oxide is held in solution under such conditions. But Shaffer holds that the power to hold cuprous oxide in solution exhibited by the filtrates above mentioned is exhibited only by blood which is not fresh. His reason for this conclusion is that the filtrates from fresh blood incubated with yeast will not hold any more cuprous oxide in solution than will an incubated suspension of yeast in pure solution, though both will hold appreciable quantities of the oxide in solution. To our minds Shaffer's experiment in this connection does not settle the point as to whether some cuprous oxide may escape precipitation from the filtrates from fresh blood. It is conceivable that both yeast or blood protein may leave a maximum of the substance in solution which is the effective agent in preventing complete precipitation of the reduced oxide. It seems that a much better criterion would have been to compare the results obtained by the Shaffer method and by some standard method, where the same sample of fresh blood was employed.

In this connection we may report two experiments, one made upon ox blood which was a few days old, the other made upon fresh ox blood. In both cases some glucose was added to the whole blood. The procedure was to divide the blood into two measured samples, one being analyzed for glucose by the Waymouth Reid method (precipitation with phosphotungstic acid, filtration, and subsequent extraction of the precipitate with boiling water, glucose being estimated in the combined filtrates by the Allihn method); the other sample of blood being heated to complete coagulation with  $\frac{N}{100}$  acetic acid, colloidal iron being then added and the glucose determined in the filtrate by direct weighing of the cuprous oxide precipitated upon boiling with Fehling's solution. This latter procedure for freeing the blood from protein was described in our previous paper on blood sugar determination, and does not differ materially from Shaffer's procedure except that he adds sodium sulphate, in addition to the colloidal iron. Sample 1 of ox blood (some days old, to which some glucose was added) gave 140 mgm. of sugar per 100 grams of blood by the Waymouth Reid method, and only 69 mgm. of glucose per 100 grams of blood when the cuprous oxide precipitated from the heat-colloidal iron filtrate was determined. Sample 2 of ox blood (fresh, but to which some glucose had been added) gave 128 mgm. of glucose per 100 grams of blood by the Waymouth Reid method, and 84 mgm. of glucose per 100 grams of blood by

precipitation of the cuprous oxide from the heat-colloidal iron filtrate. While these results are not directly applicable to the Shaffer method (because we did not use any sodium sulphate, since our procedure gave water-clear filtrates without any addition of a salt), we feel that they do call attention to the desirability of testing Shaffer's procedure by comparison of results obtained by it with some standard method upon the same samples of fresh blood for any individual species.

#### THE NEW METHOD.

The red color<sup>46</sup> obtained by heating a dextrose solution with picric acid and sodium carbonate is employed as the basis of the proposed method for the determination of blood sugar. The blood protein is removed by precipitation with picric acid,<sup>47</sup> a method which lends itself to the purpose only because of the fact that picric acid is one of the reagents of the color reaction and need not be removed from the protein-free filtrate.<sup>48</sup> The method of blood sugar determination as we use it in practice is as follows: Two cc.<sup>49</sup> of blood are aspirated through a hypodermic needle and piece of rubber tubing into an Ostwald pipette, a little powdered potassium oxalate in the tip of the pipette preventing clotting. The blood is drawn up a little above the mark and the end of the pipette is closed with the finger. After the rubber tubing and needle are disconnected, the blood is allowed to flow back to the mark and is discharged at once into a 25 cc. volumetric flask containing 5 cc. of water. The contents of the flask are shaken to insure thorough mixing and the consequent hemolysis of the blood. Then 15 cc. of saturated aqueous solution of picric acid are added, as well as a drop or two of alcohol to dispel any foam, and the contents of the flask are made up to the mark with water and then shaken. After filtra-

<sup>46</sup> The colored derivative formed is probably picramic acid.

<sup>47</sup> In a preliminary report of this method (*loc. cit.*), we used heat and colloidal iron to remove the protein. Since the early part of 1914 we have been using picric acid and find the method much improved by its use.

<sup>48</sup> Folin (*this Journal*, xvii, p. 475, 1914) employs picric acid as a precipitant for protein in blood, tissues, etc., prior to creatine and creatinine determinations.

<sup>49</sup> A smaller quantity of blood may be used if necessary.



tion 8 cc.<sup>50</sup> aliquots are measured out into large Jena test-tubes for duplicate determinations. Two cc. of saturated picric acid solution and exactly 1 cc. of 10 per cent sodium carbonate are added (as well as two glass beads and two or three drops of mineral oil), and the contents of the flask are evaporated rapidly over a direct flame until precipitation occurs. About 3 cc. of water are added, the tube is again heated to boiling to dissolve the precipitate, the contents of the tube are transferred quantitatively to a 10 cc.<sup>51</sup> volumetric flask, cooled, made up to the mark, shaken, and then filtered through cotton into the colorimeter chamber. The color is compared at once with that obtained from 0.64 mgm. of dextrose, 5 cc.<sup>52</sup> of saturated picric acid, and 1 cc. of 10 per cent sodium carbonate, when evaporated to precipitation over a free flame and diluted to 10 cc. as was the unknown, or against the picramic acid standard mentioned below.

*Calculation.* If the directions as outlined are followed exactly the calculation of the sugar present in the unknown blood sample is very simple. The original 2 cc. of blood were diluted to 25 cc., and of this amount 8 cc. were taken for a determination. In other words, the aliquot contained the equivalent of  $8/25 \times 2$  cc., or 0.64 cc. of blood. The following formula may be used to find the blood sugar content:

$$\begin{aligned} \text{Mgm. of dextrose in unknown} &= \frac{\text{reading of standard}}{\text{reading of unknown}} \times \frac{\text{mgm. of dextrose in standard.}}{\text{reading of unknown}} \\ \text{Or:} \\ \text{Mgm. of dextrose per cc. of blood} &= \frac{\text{reading of standard}}{\text{reading of unknown}} \times \frac{\text{mgm. of dextrose in standard}}{\text{cc. of blood used}} \end{aligned}$$

But the amount of dextrose in the standard is 0.64 mgm., and the amount of blood used is 0.64 cc. Consequently the second fraction equals unity and the equation is simplified.

<sup>50</sup> When only a small amount of blood is available, satisfactory results may be obtained by using a single larger aliquot.

<sup>51</sup> In case of hyperglycaemia the final volume of the reaction fluid is made 25 cc. or 50 cc., and the results are accordingly multiplied by 2.5 or 5.0.

<sup>52</sup> It was found that provided the equivalent of 4 cc. of saturated picric acid were present, the quantity of this reagent had no influence on the amount of color produced.

$$\text{Mgm. of dextrose per cc. of blood} = \frac{\text{reading of standard}}{\text{reading of unknown}}$$

The per cent of blood sugar is, of course, 0.1 of the figure thus obtained.

*Permanent standard.* A solution of picramic acid<sup>52</sup> makes a very satisfactory permanent standard. The color is identical in quality with that resulting from the alkaline-picrate-sugar reaction. Furthermore this solution of picramic acid keeps perfectly. Following is the formula of the permanent standard:

Picramic acid.....	0.064 gm.
Sodium carbonate (anhydrous).....	0.100 gm.
Water to make.....	1000 cc.

Dissolve the picramic acid with the aid of heat in 25 to 50 cc. of distilled water which has been made alkaline with the sodium carbonate. Cool and dilute to one liter. This solution has the same intensity of color as that obtained by the proposed method with 0.64 mgm. of sugar when the final volume of the reaction fluid is made 10 cc. We have used only two samples of picramic acid, and it is quite possible that other samples might give a solution of different color intensity. For this reason the picramic acid solution should be standardized before being used.

*Accuracy of proposed method.* The accuracy and delicacy of this color reaction for the determination of blood sugar was shown by the analysis of pure sugar solutions (Table I). Dehn and Hartman<sup>54</sup> have recently published a method of sugar determination, the basis of which is the color reaction used by us.<sup>55</sup> Their results

<sup>52</sup> Picramic acid was formerly obtainable only in the European market. At our suggestion the J. T. Baker Chemical Company, of Phillipsburg, New Jersey, are manufacturing picramic acid, and have placed it upon the market in this country. We have examined a sample of their product and found it wholly satisfactory.

<sup>54</sup> W. M. Dehn and F. A. Hartman: *Jour. Am. Chem. Soc.*, xxxvi, p. 403, 1914.

<sup>55</sup> Attention should be called to the fact that we first reported our blood sugar method before the Society for Experimental Biology and Medicine on Dec. 17, 1913. The paper of Dehn and Hartman (*loc. cit.*) appeared in February, 1914. These authors made no attempt to use the method for blood sugar determinations. We had used the color reaction successfully for the determination of pure sugar solutions since the early part of 1913.

## Estimation of Sugar in Blood

TABLE I.

*Analysis of pure dextrose solutions by the proposed methods.*

DEXTROSE	
Taken:	Found:
mgm.	mgm.
1.00	1.00
0.90	0.90
0.80	0.79
1.00	1.00
0.90	0.89
0.80	0.795
2.00	2.00
5.00	5.00

likewise demonstrate the accuracy of the method. Our method has an accuracy of from 1 to 2 per cent of the amount of sugar present.

When we first used picric acid we collected the blood directly into a saturated picric acid solution, the results obtained agreeing closely with those when our heat-colloidal iron method of protein removal was employed. We soon found, however, that, if hemolysis occurred previous to precipitation of the blood protein, higher figures resulted. Table II shows comparable determinations of blood sugar by the different modifications of the colorimetric method just mentioned.

The consistently higher figures obtained by the method as described in this paper (precipitation of the blood protein with picric acid after hemolysis) should be noted. Evidently unless hemol-

TABLE II.

*Comparison of the heat-colloidal iron and picric acid methods of protein precipitation as a preliminary to the determination of blood sugar by the new colorimetric method.*

*The figures give dextrose per cent.*

HEAT-COLLOIDAL IRON (BLOOD COLLECTED DIRECTLY INTO HOT $\frac{N}{100}$ ACETIC ACID)	PICRIC ACID (NO HEMOLYSIS)	PICRIC ACID (HEMOLYSIS PREVIOUS TO PRE- CIPITATION OF BLOOD PROTEIN)
0.164	0.166	
0.119	0.118	
0.105	0.105	
0.361	0.361	
0.094		0.101
	0.124	0.130
	0.153	0.163

TABLE III.

*Comparison of the gravimetric method of Waymouth Reid for the determination of blood sugar with the new colorimetric method.*

BLOOD	DEXTROSE		
	Gravimetric method of Reid	Colorimetric method (heat-colloidal iron precipitation)*	Colorimetric method (picric acid precipitation after hemolysis)
	per cent	per cent	per cent
Ox.....	0.0588	0.0552 [corrected 0.0585]	
Ox.....	0.0596	0.0563 [corrected 0.0597]	
Human.....	0.0985		0.098
Human.....	0.119		0.119
Human.....	0.122		0.122
Human.....	0.111		0.108

\*As we have already pointed out, this method of blood protein precipitation yields results 5 to 7 per cent too low (cf. Table II). Consequently we have made [in brackets] a 6 per cent correction.

ysis takes place previous to precipitation of the protein, some sugar is lost by inclusion in the red blood cells.

Table III gives a comparison of blood sugar determinations by the proposed method and by the gravimetric method of Waymouth Reid. A very close agreement in the results obtained by the two methods is seen.<sup>56</sup>

TABLE IV.

*Showing the complete recovery by the new method of dextrose added to blood.*

	DEXTROSE	
	Theoretical	Found
	per cent	per cent
Ox blood I (original analysis).....		0.0543
Ox blood I + 0.5 mgm. dextrose per cc.....	0.1043	0.1048
Ox blood I + 1 mgm. dextrose per cc.....	0.1543	0.1548
Ox blood II (original analysis).....		0.0710
Ox blood II + 1 mgm. dextrose per cc.....	0.1710	0.1720
Ox blood (+ dextrose) III (original analysis).....		0.1580
Ox blood (+ dextrose) III + 0.5 mgm. dextrose per cc..	0.2080	0.2080
Ox blood (+ dextrose) III + 1 mgm. dextrose per cc....	0.2580	0.2580

<sup>56</sup> When our method was first devised, we feared that it might give slightly too high values for blood sugar, owing to the presence in blood of creatinine, or of other compounds giving the same color reaction as that given by the

\* When dextrose was added to blood it could be quantitatively recovered by the new colorimetric method as is shown in Table IV.

*Normal blood sugar content.* An examination of the bloods of healthy persons has shown a normal blood sugar content of 0.09 to 0.11 per cent (average = 0.1 per cent). This figure agrees with that reported in the literature by the majority of authors.

glucose. The results do not, however, show any tendency to be higher than those obtained by other standard methods. Hence we may infer that the traces of creatinine present in blood are not sufficient to affect the color values obtained.

## THE SYNTHESIS OF HIPPURIC ACID IN EXPERIMENTAL TARTRATE NEPHRITIS IN THE RABBIT.<sup>1</sup>

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(Received for publication, November 19, 1914.)

The object of this investigation was to determine whether nephritis influences the synthesis of hippuric acid in the rabbit.

The chief conditions affecting the synthesis of hippuric acid are known from the work of Bunge and Schmiedeberg<sup>2</sup> and the later experiments of Hoffmann,<sup>3</sup> W. Koch,<sup>4</sup> and Bashford and Cramer.<sup>5</sup> The place of hippuric acid synthesis in the dog is definitely believed to be the kidney, but in the case of the rabbit the evidence is somewhat conflicting.

Salomon,<sup>6</sup> using the analytical method of Bunge and Schmiedeberg, showed that the removal of the kidneys of the rabbit did not prevent the synthesis of hippuric acid. The necessary glycocoll and benzoic acid were introduced into the stomach of the rabbit after the kidneys had been extirpated, and hippuric acid was found in the blood, liver, and muscles. He found that tying off the ureters did not cause any increase of hippuric acid in the blood, muscles, or liver over that found when the kidneys were extirpated. Salomon concluded that the formation of hippuric acid in the rabbit occurred in other places than the kidney.

Jaarsveld and Stokvis<sup>7</sup> extirpated one kidney of a rabbit, tied off the other at the hilus for 1 hour and 20 minutes, and then introduced 1 gm. of benzoic acid into the stomach. The rabbit died in 22 hours, and hippuric acid could be found neither in the blood nor in the urine. The same result

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<sup>1</sup> Aided by a grant from the Research Fund of the University of Minnesota.

<sup>2</sup> G. Bunge and O. Schmiedeberg: *Arch. f. exp. Path. u. Pharmacol.*, vi, p. 233, 1876.

<sup>3</sup> A. Hoffmann: *Arch. f. exp. Path. u. Pharmacol.*, vii, p. 233, 1877.

<sup>4</sup> W. Koch: *Arch. f. d. ges. Physiol.*, xx, p. 64, 1879.

<sup>5</sup> E. F. Bashford and W. Cramer: *Ztschr. f. physiol. Chem.*, xxxv, p. 324, 1902.

<sup>6</sup> W. Salomon: *Ztschr. f. physiol. Chem.*, iii, p. 365, 1879.

<sup>7</sup> G. J. Jaarsveld and B. J. Stokvis: *Arch. f. exp. Path. u. Pharmacol.*, x, p. 268, 1878.

was obtained when both kidneys were removed; no hippuric acid could be found in the blood.

A rabbit, which had previously been fed benzoic acid, was injected subcutaneously with a 50 per cent glycerine solution, which produced a marked hemoglobinuria. The urine was analyzed for hippuric and benzoic acids to see what effect the injury to the kidneys so produced would have on the synthesis. In most of the experiments of this kind the percentage of the total benzoic acid in the form of hippuric acid was much less than the percentage of the free benzoic acid, but in one experiment it was much more. In the case of normal rabbits Jaarsveld and Stokvis found nearly all of the ingested benzoic acid in the form of hippuric acid in the urine; but in all experiments except one they were able to find weighable quantities of free benzoic acid. The glycerine injections prevented the synthesis to a large extent.

A rabbit, which had been given benzoic acid by stomach tube, was killed 5½ hours later by bleeding. 0.115 gm. of free benzoic acid and 0.058 gm. of hippuric acid were found in the stomach and small intestine. 0.135 gm. of free benzoic acid and no hippuric acid were found in the blood, 0.016 gm. of free benzoic acid and traces of hippuric acid in the urine, and neither free benzoic acid nor hippuric acid in the large intestine.

The kidneys of a normal rabbit were extirpated, and 1 gm. of benzoic acid was introduced into the stomach. The contents of the stomach and small intestine were analyzed a short time after and 0.114 gm. of free benzoic acid and 0.082 gm. of hippuric acid were found.

Jaarsveld and Stokvis concluded that in the rabbit the synthesis takes place in more than one part of the body; namely, in the kidneys, liver, and small intestine.

Jaarsveld and Stokvis used Bunge and Schmiedeberg's analytical method for blood and tissue analysis, and their own modification of this method for the urine analysis.

It is doubtful, however, whether Jaarsveld and Stokvis really produced a severe tubular nephritis by the injection of glycerine. They described a marked hemoglobinuria in every case. Potter and one of us (Bell) have shown that hemoglobin is eliminated by the convoluted tubules of the rabbit kidney, and that when these structures are all destroyed or severely injured only a very little hemoglobin is excreted in the urine. The marked hemoglobinuria produced by glycerine does not signify the presence of severe injury to the kidney. It is due to the liberation of a large amount of hemoglobin in the circulating blood.

Weyl and Anrep<sup>8</sup> found that, in rabbits in which fever had been produced by the injection of pus, the ability to synthesize hippuric acid was greatly diminished. With dogs this was also true, but to a much less extent.

Van de Velde and Stokvis<sup>9</sup> found that after the injection or feeding of benzoate to rabbits all the benzoic acid of the urine was present as hippuric

<sup>8</sup> Th. Weyl and B. von Anrep: *Ztschr. f. physiol. Chem.*, iv., p. 169, 1880.

<sup>9</sup> A. van de Velde and B. J. Stokvis: *Arch. f. exp. Path. u. Pharmacol.*, xvii, p. 189, 1883.

acid, provided the urine was acid. If the urine was alkaline, as much as 50 per cent might be present as free benzoic acid. These authors called attention to the fact that hippuric acid was very easily hydrolyzed in alkaline media. This fact was apparently not known to many of the later investigators, for in many of the later methods for hippuric acid estimation the evaporation of the liquid containing it with alkali is recommended.

We have found that, after the injection of sodium benzoate, rabbit urine may contain large amounts of unconjugated or free benzoic acid (as the Na salt if the urine is alkaline, as it usually is). Other investigators have also found this to be the case (Wiener,<sup>10</sup> van de Velde and Stokvis, Jaarsveld and Stokvis). We call attention to this fact because it has been assumed from time to time that rabbit urine under these conditions contained so little benzoic acid, if any at all, that it was not necessary to consider it.

Howard B. Lewis,<sup>11</sup> in a study of the relation of the hippuric acid nitrogen to urea in rabbit urine, neglected to take account of the free benzoic acid present in the urines which he analyzed for hippuric acid on the assumption that it was not there; so that his figures for hippuric acid and the nitrogen present as hippuric acid cannot be accepted, since it is quite possible that a large part of the so called hippuric acid (total benzoic acid) might have been present as free benzoic acid.

#### METHODS.

The experimental work of Underhill, Wells, and Goldschmidt<sup>12</sup> has given us a method for the production of a severe tubular nephritis with a minimum of glomerular injury. The nephritis was produced in rabbits by the subcutaneous injection of small amounts of racemic tartaric acid.

This is the procedure which we have used in our experiments to produce nephritis. By administering large amounts of water by stomach tube we have found that it is often possible to get considerable excretion of urine from severely injured kidneys. If the animals are not forcibly watered the amount of urine obtained is usually too small for accurate analysis.

The physiologic condition of the kidneys was determined by the phenol-sulphonaphthalein test introduced by Rowntree and Geraghty.<sup>13</sup> Rowntree and Fitz<sup>14</sup> have produced abundant clinical evidence that this test is an accurate index of the functional capacity of the kidney. A very low phthalein excretion was always associated with a severe clinical case of nephritis. Their work has been corroborated by a large number of observers.

<sup>10</sup> H. Wiener: *Arch. f. exp. Path. u. Pharmacol.*, xl, p. 313, 1898.

<sup>11</sup> H. B. Lewis: *this Journal*, xvii, p. 505, 1914.

<sup>12</sup> F. P. Underhill, H. G. Wells, and S. Goldschmidt: *Jour. Exper. Med.*, xviii, p. 322, 1913.

<sup>13</sup> L. G. Rowntree and J. T. Geraghty: *Jour. Pharm. and Exper. Therap.*, i, p. 579, 1909-10.

<sup>14</sup> L. G. Rowntree and R. Fitz: *Arch. Int. Med.*, xi, p. 258, 1913.



In rabbits Potter and one of us (Bell)<sup>15</sup> found, by killing the animals at the end of the test and examining the kidneys, that a trace or a zero phthalein excretion for 2 hours is associated invariably with a severe tubular injury, and that marked granular degeneration is present when the phthalein excretion is as low as 5 or 6 per cent.

The work of Rowntree and Geraghty, as well as that of Potter and Bell, has proved that phenolsulphonephthalein is excreted by the tubules and not by the glomeruli. A decrease of phthalein excretion corresponds to tubular injury.

In addition to the functional test with phthalein, two of our animals were killed at the end of the experiment, and the gross and microscopic changes in the kidneys were studied. The urine was taken from the collecting jar at 24 hour intervals. After the injection of tartrate it was collected by catheter, so as to separate the 24 hour specimens sharply. The use of the catheter is the more necessary since retention of urine is common in the nephritic animal.

A preliminary test with phenolsulphonephthalein was made at the beginning of the experiment. A known amount of sodium benzoate was injected subcutaneously daily and the urine analyzed for a few days before the tartrate was given. It was found that without the subcutaneous injection of benzoate there was a larger error in estimating the benzoic and hippuric fractions of the small amount of total benzoic acid normally excreted by the rabbit. The rabbits were then injected intramuscularly with 0.3 to 0.7 gm. of racemic tartaric acid dissolved in 10 to 20 cc. of water and neutralized with sodium carbonate. After several hours the phthalein test was made. If the phthalein excretion was very low or suppressed, the daily injections of sodium benzoate were continued and the urine was collected by catheter at 24 hour intervals. The animals were watered by stomach tube, 50 to 100 cc. daily. If not forcibly watered the rabbit will usually drink very little, and the amount of urine passed may be too small for satisfactory analysis. The benzoate also acts as a diuretic. Considerably more urine is usually obtained as a result of its use. One must wait several hours after the tartrate injection before benzoate is administered, since the latter seems to prevent the development of a severe nephritis if injected too soon.

The rabbits were fed on carrots exclusively. They were allowed all they would eat of this food. A dish of water was kept in the cage constantly. Each day about 0.5 cc. of a thymolchloroform solution was put into the collecting jar for the urine as an antiseptic. During the earlier part of the work enough dilute hydrochloric acid was put into the jar to more than neutralize the alkali of the urine; but this procedure was soon discontinued since it seemed unnecessary. The urines were analyzed immediately so that there was no appreciable loss of ammonia.

We have found that the Folin and Flanders<sup>16</sup> method for the determina-

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<sup>15</sup> A. C. Potter and E. T. Bell: *Am. Jour. Med. Sc.*, 1915, (in press).

<sup>16</sup> O. Folin and F. F. Flanders: *this Journal*, xi, p. 257, 1912.

tion of hippuric acid was very satisfactory, and it is the method that we have used for all our analyses.

Folin and Flanders<sup>17</sup> were able to extract benzoic acid quantitatively from cranberries and tomato ketchup, and to determine accurately the amount of this acid in the extract by titration against tenth normal sodium ethylate, as they determined the liberated benzoic acid in their hippuric acid method. The method that we have used for free benzoic acid is based on the above method, and is as follows:

*Method for the determination of free benzoic acid in rabbit urines.* 25 or 50 cc. of the rabbit urine are transferred by pipette into a small beaker, a drop of dilute alizarine red indicator is added, showing the usual alkalinity of the urine due to the presence of sodium carbonate, and concentrated hydrochloric acid added from a burette drop by drop until the solution becomes acid. The solution is now boiled for a minute to remove the excess of carbon dioxide, as this has been found to interfere materially with the accuracy of the method.

This boiling must not be continued or an appreciable amount of hippuric acid will be hydrolyzed and determined with the benzoic acid; but, as will be shown, boiling for this short time will not split a measurable quantity of hippuric acid. The cool solution is now transferred to a separatory funnel by means of enough water to bring the total volume up to 100 cc., 0.5 cc. of concentrated hydrochloric acid is added to make the solution distinctly acid, and the solution saturated with ammonium sulphate by dissolving it in 55 gm. of the latter.

The saturated liquid is now extracted with neutral, alcohol-free chloroform in one 50 cc., one 35 cc., and two 25 cc. portions, as in the Folin-Flanders method for hippuric acid. The chloroform extract is washed with three 100 cc. portions of saturated sodium chloride solution containing 0.5 cc. of hydrochloric acid in 1000 cc. of solution. This washing is carried out in a second separatory funnel. The washed extract is titrated against tenth normal sodium ethylate with phenolphthalein as indicator. Duplicate determinations will give titration figures varying by not more than 0.05 cc., if carefully made.

Special experiments showed that the removal of liberated carbon dioxide from the acidified rabbit urine before extraction with chloroform is absolutely essential.

The following experiment shows that under the prescribed conditions only a negligible hydrolysis of hippuric acid occurs. 0.15 gm. of crystalline hippuric acid was dissolved in about 25 cc. of hot water, one drop of alizarine red indicator added, and the solution made distinctly acid by the addition of two drops of concentrated hydrochloric acid. The solution was boiled for a minute and allowed to cool, when the hippuric acid separated out as crystals. On extracting and titrating the extract, two drops of tenth normal ethylate were more than sufficient to neutralize all the benzoic acid that had been split from the hippuric acid.

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<sup>17</sup> Folin and Flanders: *Jour. Am. Chem. Soc.*, xxxiii, p. 1622, 1911.

To prove that benzoic acid could be added to rabbit urine and quantitatively recovered by this method, 5 cc. of a solution of sodium benzoate having an acid equivalent of 12.25 cc. of tenth normal ethylate were added to 25 cc. of rabbit urine and the urine was analyzed.

25 cc. of rabbit urine contained benzoic acid, the equivalent of 2.70 cc.  $\frac{N}{10}$  ethylate.

25 cc. of rabbit urine plus 5 cc. of sodium benzoate contained benzoic acid, the equivalent of 14.90 cc.  $\frac{N}{10}$  ethylate.

Sodium benzoate by difference, 12.20 cc.  $\frac{N}{10}$  ethylate.

Sodium benzoate by analysis, 12.25 cc.  $\frac{N}{10}$  ethylate.

The difference of 0.05 cc. is negligible.

*Rabbit F.* The animal was injected every day at 11 a.m. with 20 cc. of a sodium benzoate solution which contained the equivalent of 0.630 gm. of

TABLE 1.

*Rabbit F.*

DAYS	VOLUME OF URINE	RACEMIC TARTARIC ACID INJECTED	PHENOL SULPHONE- PHTHALEIN TEST	BENZOIC ACID EQUIVA- LENT TO SODIUM BEN- ZOATE INJECTED	TOTAL NITROGEN	FREE BENZOIC ACID	HIPPURIC ACID	HIPPURIC ACID NITRO- GEN	HIPPURIC ACID NITRO- GEN OF TOTAL NITRO- GEN	HIPPURIC ACID OF TO- TAL BENZOIC ACID
	cc.			gm.	gm.	gm.	gm.	gm.	per cent	per cent
1	71.0		Normal	0.630	1.107	0.076	0.559	0.044	3.9	83.5
2	115.0			0.630	1.220	0.045	1.043	0.082	6.7	94.3
3	95.0			0.630	0.702	0.033	0.834	0.065	9.3	94.5
4	105.0			0.630	1.246	0.042	0.834	0.065	5.2	93.1
5	115.0			0.630	0.829	0.046	0.817	0.064	7.2	92.4
6	208.0			0.630	1.167	0.093	0.951	0.074	6.4	87.4
7	135.0		Normal	0.630	0.556	0.131	0.736	0.058	10.3	79.3
8	157.0	Feb. 2, 11 a.m., 0.4 gm.		0.630	0.677	0.055	0.959	0.075	11.1	92.3
9	29.0	Feb. 3, 5 p.m., 0.6 gm.		0.630	0.088	0.015	0.284	0.022	25.3	93.0
10	108.0		Feb. 4, less than 2%	0.630	0.442	0.049	0.541	0.042	9.6	88.4
11	160.0		Feb. 5, less than 2%	0.630	0.999	0.151	0.886	0.069	6.9	80.1
12	187.0		Feb. 7, 5.7%	0.630	1.614	0.415	0.701	0.055	3.4	53.5

benzoic acid, as determined by analysis. On the day that the tartaric acid was given, the injection of the sodium benzoate was postponed until 4 p.m. It had been found that, if the benzoate was given at the same time as the tartaric acid, the latter was not so efficient in its action on the kidneys. The results of the analyses and of the phthalein tests are recorded in Table I.

At autopsy the kidneys were considerably enlarged and cloudy in appearance. Crushed pieces of fresh tissue showed all the tubules to be very granular. Sections in paraffin showed an irregularly granular fragmented cytoplasm. No necrosis was present. There was some fat in the tubules.

The percentage of the total benzoic acid present in the urine as hippuric acid remained remarkably constant throughout the experiment. In the period before the injection of tartaric acid the average percentage of hippuric acid was 89.7 per cent as compared with 78.7 per cent during the period when the phthalein test indicated severely damaged kidneys.

It is interesting to note that the total amount of benzoic acid recovered in 12 days was 7.296 gm. as compared with 7.560 gm. injected, although a small amount of urine was lost on February 4. On this day the total amount of benzoic acid recovered was approximately a third of the average normal quantity, and the hippuric acid nitrogen was 25 per cent of the total nitrogen.

TABLE II.

*Rabbit G.*

DAYS	VOLUME OF URINE	RACEMIC TARTARIC ACID INJECTED	PHENOLSULPHONE- PHTHALEIN TEST	BENZOIC ACID EQUIVA- LENT TO SODIUM BEN- ZOATE INJECTED	TOTAL NITROGEN	FREE BENZOIC ACID	HIPPURIC ACID	HIPPURIC ACID NITRO- GEN	HIPPURIC ACID NITRO- GEN OF TOTAL NITRO- GEN	HIPPURIC ACID OF TO- TAL BENZOIC ACID
	cc.			gm.	gm.	gm.	gm.	gm.	per cent	per cent
1	39.0			0.630	0.201	0.040	0.107	0.008	4.1	64.0
2	270.0			0.630	1.146	0.436	0.257	0.020	1.7	28.6
3	277.5			0.630	1.455	0.165	0.628	0.053	3.6	73.3
4	364.0			0.630	1.171	0.398	0.358	0.028	2.4	38.0
5	268.0	Feb. 14, injection		0.630	0.982	0.456	0.371	0.029	3.0	35.8
6	None			0.630						
7	80.0		Complete suppres- sion	0.630	0.258	0.103	0.091	0.007	2.7	37.4
8	197.0		Complete suppres- sion	0.630	0.803	0.166	0.328	0.026	3.2	57.3
9	341.0			0.630	0.990	0.872	0.324	0.025	2.6	20.1

*Rabbit G.* The conditions of this experiment were similar to those of the preceding experiment. The results are recorded in Table II.

On the seventh and eighth days there was complete phthalein suppression, and there was a trace of albumin in the urine of these days. This was not removed from the samples of urine that were analyzed for hippuric acid, although if present in any quantity it should be removed; for, on treating albumin as is required by the hippuric acid method, a certain quantity of benzoic acid can be formed from it, as is shown by the following experiment.

5 gm. of Merck's egg albumin were boiled 4.5 hours with 25 cc. of concentrated nitric acid and 23 cc. of water in the presence of 0.2 gm. of cupric nitrate. The chloroform extract of this reaction mixture required for neutralization 18.40 cc. of tenth normal sodium ethylate, equivalent to 0.2294 gm. of benzoic acid. The acid was identified as benzoic acid.

It seems to be characteristic of rabbit urines after tartaric acid injections that they are thin and watery and usually contain slight traces of albumin. In the experiments with Rabbit G there was so little albumin present that we are certain that it could not have appreciably altered the figures for benzoic and hippuric acids.

Rabbit G gradually recovered, although there was complete suppression of phthalein excretion for two days.

This experiment agrees with that on Rabbit F, that severe renal injury did not cause any significant change in the relations between the free and combined benzoic acids present in the urine. The average percentage of the total benzoic acid present as hippuric acid for the period just before the injection of tartaric acid (February 10 to February 15) was 47.5 per cent, and for the period during which there were two days of complete suppression of the phthalein the percentage was 38.2 per cent (February 15 to the end of the experiment).

Of the 5.670 grams of benzoic acid injected during the 9 days 4.348 grams were recovered.

*Rabbit H.* The experiment with this animal was conducted in the same way as the preceding, except that there was a shorter period before the injection of tartaric acid. On the fifth day the animal was killed.

The kidneys were found greatly enlarged and cloudy. Crushed pieces of fresh tissue showed marked granular degeneration and considerable fat. Paraffin sections showed necrosis of the zone of convoluted tubules adjacent to the capsule. The tubules in other situations showed pronounced granular degeneration but no necrosis. The glomeruli were apparently not injured. This represents therefore a severe tubular nephritis.

The average percentage of the total benzoic acid present as hippuric acid for the 2 days previous to the tartaric acid injections was 77.8 per cent; and

TABLE III.  
*Rabbit H.*

DAYS	VOLUME OF URINE	RACEMIC TARTARIC ACID INJECTED	PHENOLSULPHONE-PHTHALEIN TEST	BENZOIC ACID EQUIVALENT TO SODIUM BENZOATE INJECTED	TOTAL NITROGEN	FREE BENZOIC ACID	HIPPURIC ACID	HIPPURIC ACID NITROGEN	HIPPURIC ACID NITROGEN OF TOTAL NITROGEN	HIPPURIC ACID OF TOTAL BENZOIC ACID
	cc.			gm.	gm.	gm.	gm.	gm.	per cent	per cent
1	118.0	Feb. 25, 11 a.m., injection	Trace	0.630	1.389	0.082	1.019	0.080	5.7	89.4
2	108.0			0.630	0.624	0.212	0.610	0.048	7.6	66.2
3	29.1			0.630	0.087	0.042	0.109	0.009	9.8	63.8
4	118.0			0.630	0.472	0.152	0.310	0.024	5.1	58.1
5	258.0			0.630	1.237	0.128	0.827	0.065	5.2	81.5

for the 3 days after the injections, during 2 of which there was complete suppression of the phthalein, the average percentage was 67.8 per cent. This experiment was also in complete agreement with the preceding ones.

Of the 3.15 gm. of benzoic acid injected during the 5 days, 2.578 gm. were recovered.

TABLE IV.  
*Rabbit K.*

DAYS	VOLUME OF URINE	RACEMIC TARTARIC ACID INJECTED	PHENOLSULPHONE-PHTHALEIN TEST	BENZOIC ACID EQUIVALENT TO SODIUM BENZOATE INJECTED	TOTAL NITROGEN	FREE BENZOIC ACID	HIPPURIC ACID	HIPPURIC ACID NITROGEN	HIPPURIC ACID NITROGEN OF TOTAL NITROGEN	HIPPURIC ACID OF TOTAL BENZOIC ACID
	cc.			gm.	gm.	gm.	gm.	gm.	per cent	per cent
1	71.5	Sept. 22, 4.30 p.m., 0.5 gm.	Suppression	1.14	0.432	0.452	0.325	0.025	5.8	32.9
2	345.0			1.14	1.368	0.464	1.070	0.084	6.1	61.1
3	197.0			None	1.595	0.155	1.848	0.144	9.0	89.0
4	Lost			1.14						
5	66.0			1.14	0.195	0.029	0.317	0.025	12.7	91.7

*Rabbit K.* The diet of this rabbit consisted entirely of milk, of which it was given all that it would drink. The results are recorded in Table IV.

The percentage of hippuric acid of the total benzoic acid shows a steady increase from 32.9 to 91.7 per cent. On the day when there was practically complete suppression of phthalein the percentage of hippuric acid was the highest, 91.7 per cent.

This experiment also shows that tartrate nephritis does not alter the mechanism of hippuric acid synthesis.

#### DISCUSSION AND SUMMARY.

Our experiments include cases of severe acute nephritis (Rabbits G and H) as well as cases of moderate intensity. In Rabbit H the functional test was checked by the autopsy findings. There is no doubt that a complete suppression of phenolsulphonaphthalein is always associated with severe injury to the convoluted tubules. Reference to the accompanying tables will show, however, that there was never any definite interference with the hippuric acid synthesis during the nephritis. We must therefore conclude that severe injury of the convoluted tubules does not affect the synthesis of hippuric acid.

The glomeruli and the collecting tubules are not seriously injured in tartrate nephritis. If hippuric acid is synthesized in the kidney of the rabbit, this synthesis must be accomplished under the influence of either the degenerating cells of the convoluted tubules, or the cells of the glomeruli or collecting tubules. We are inclined to the view that the synthesis of hippuric acid does not occur in the kidney of the rabbit.

## THE SOLUBLE POLYSACCHARIDES OF LOWER FUNGI.

### III. THE INFLUENCE OF AUTOLYSIS ON THE MYCODEXTRAN CONTENT OF *ASPERGILLUS NIGER*.

By ARTHUR W. DOX.

(From the Chemical Section of the Iowa Agricultural Experiment Station.)

(Received for publication, November 24, 1914.)

The first two papers<sup>1</sup> of this series describe the preparation and properties of the two new carbohydrates, mycodextran and mycogalactan, from lower fungi. The material used in preparing these substances was the dried fungus from cultures that had just reached maturity but had not undergone autolysis. It was stated that attempts to prepare these polysaccharides from cultures that had autolyzed for six weeks were unsuccessful. The present work was undertaken with the view of determining the quantitative yield of mycodextran from cultures grown under the same conditions for different periods of time, and of following the variations due to autolysis.

A large number of liter Erlenmeyer flasks, each containing 200 cc. of Raulin's medium, were sterilized and inoculated with spores of *Aspergillus niger*. At different stages of growth or autolysis, indicated in the table below, the fungus was removed from five flasks, washed with distilled water, and dried collectively at 100°. After drying, the mold was ground to a fine powder and extracted three times for a half hour with successive portions of 100 cc. of boiling water. A clear filtrate was obtained in each case, from which the mycodextran generally separated out in a white flocculent form. Three extractions were considered sufficient since a fourth extraction gave only a trace of mycodextran. The crude mycodextran was filtered with suction on a hardened filter, washed with cold water until the color had been removed, then

<sup>1</sup> A. W. Dox and R. E. Neidig: this *Journal*, xviii, pp. 167-175, 1914; xix, pp. 235-237, 1914.



## 84 Mycodextran Content of *Aspergillus niger*

washed several times with alcohol, and finally dried to constant weight at 100°. The product was a hard white crust which was easily removed from the paper. The results obtained as above are recorded in the following table.

AGE OF CULTURE	WEIGHT OF DRY FUNGUS	MYCODEXTRAN (DRY FUNGUS)	
dys.	gm.	gm.	per cent
3	13.648	Trace	
5	20.096	0.177	0.88
7	13.977	0.666	4.76
14	8.026	0.589	7.34
21	7.537	0.570	7.56
28	6.915	0.505	7.30
35	7.002	0.549	7.84
42	6.734	0.546	8.11
49	6.441	0.544	8.45
56	6.171	0.523	8.47
63	6.331	0.544	8.59
142	5.938	0.546	9.19

In the three day cultures a dense white mycelium had completely covered the surface of the medium, but spores had not yet begun to form. The five day cultures showed quite a few black spores and a great number of yellow spores in which the pigment had not yet developed. The week old cultures were jet black on the surface. At this stage autolysis had already begun, as indicated by the decrease in weight of the dry fungus, and from this point on a gradual decrease in weight occurred, similar to that reported for *Aspergillus fumigatus* in another paper.<sup>2</sup> As autolysis progressed, the hot water extract became lighter in color, while, on the other hand, the medium became darker. Much to our surprise, the yield of mycodextran was found to be approximately constant for a given amount of culture medium. The percentage yield, of course, increased as the weight of the fungus diminished. Our previous failure to obtain mycodextran from cultures of *Penicillium expansum* that had autolyzed for six weeks, we are at present unable to explain. In the light of this later work it appears probable that mycodextran forms an integral part of the mature fungus and does not undergo autolysis.

<sup>2</sup> A. W. Dox: this *Journal*, xvi, p. 483, 1913-14.

There is apparently a relationship between the presence of spores and the occurrence of mycodextran. From the three day cultures, which were perfectly white, the merest trace of mycodextran was obtained, whereas the five day cultures, in which spores were beginning to develop, showed an appreciable amount of this substance, and all the cultures that had grown seven days or longer and were black with spores gave the maximum yield. Since the spores retain their vitality for a long time, it is not likely that they undergo autolysis. The fairly constant yield of mycodextran might, therefore, be explained by assuming that this substance occurs only in the spores. Cramer<sup>3</sup> obtained a yield of 17 per cent of "spore starch" from the spores of *Penicillium glaucum*. This spore starch, we have reason to believe, was probably an impure preparation of mycodextran contaminated by some other carbohydrate giving the iodine reaction. When *Aspergillus niger* is grown on a fluid medium, such as Raulin's, the surface collects considerable moisture, and a separation of the spores from the hyphae is a difficult matter. Our evidence in favor of the assumption that mycodextran is localized in the spores of this organism is, therefore, entirely presumptive, until we succeed in separating a sufficient quantity of spores with which to make an extraction.

For preparing mycodextran in quantity, cultures that have undergone autolysis are preferable. Not only can the maximum yield be obtained from such cultures, but the product is more free from contaminating substances, and, therefore, more easily purified.

Whether or not mycodextran occurs as such in the fungus cannot be definitely stated at this time. The method of extraction, viz., boiling with water, might, of course, bring about a partial hydrolysis of some mother-substance, resulting in the formation of this peculiar polysaccharide.

<sup>3</sup> E. Cramer: *Arch. f. Hyg.*, xx, p. 197, 1894.



## ON THE FAT IN THE BLOOD IN A CASE OF LIPAEMIA.

By C. G. IMRIE.

(*From the Department of Pathological Chemistry of the University of Toronto.*)

(Received for publication, November 28, 1914.)

A patient was admitted into the Toronto General Hospital in March, 1914, who up to that day had been at work as a labourer, and eight hours after admission died in a state of coma.

At the autopsy it was observed that the blood that escaped in the first incision was remarkably pale in colour, appearing like cream on dividing vessels in the least dependent parts of the cadaver; in more dependent parts it was redder, but still evidently rich in fat. In the pancreas a nodular projection was found in front of the spinal column, and extensive induration, interlobular in distribution and of a more chronic character, stretched throughout the tail of the organ. A full account of the microscopical anatomy of this, as well as of a remarkable condition in the spleen of this patient, is given elsewhere by Prof. J. J. Mackenzie.<sup>1</sup> Blood was collected from the heart and great vessels of the thorax, and urine from the bladder.

In the urine sugar was found amounting to 6.2 per cent; and from 50 cc., acidified with sulphuric acid and saturated with ammonium sulphate, ether in a continuous extraction apparatus extracted a laevorotary acid which in its rotatory power was equivalent to 0.67 per cent of  $\beta$ -hydroxybutyric acid in the urine. The ammonia coefficient was 11.9. In addition, ether extracted from the urine 0.1 per cent of acids insoluble in water, the molecular weight of which by titration was found to be 282, and which in other ways too appeared to be higher fatty acids. With them was obtained also a trace of cholesterol amounting to about 3 mgm. from 50 cc. of urine.

The blood was put in a separating funnel and the creamy serum separated from the corpuscles. Its specific gravity at 16.5°C.

<sup>1</sup> J. J. Mackenzie: unpublished report.

## 88      Fat in the Blood in a Case of Lipaemia

was 1014. Of this serum 109.5 grams were treated with 5 cc. of 40 per cent potash and about 100 cc. of petroleum ether and as much alcohol. After being shaken, the clear petroleum solution was removed and the shaking was repeated three times with fresh portions of petroleum ether. A fifth portion of petroleum ether used in the same way was found to have removed only 2.5 mgm. of soluble material. The mixed petroleum solutions were made up to 250 cc. In a portion of this the dissolved matter was determined to be 14.06 per cent of the serum by weight. This residue was saponified with alcoholic potash and the alkaline solution shaken with petroleum ether several times. Unsaponifiable substances thus removed amounted to 1.5 per cent of the serum by weight.

The alkaline serum was then made acid and again shaken with petroleum ether, which now removed a further quantity of soluble matter, fatty acids, originally present in the serum as soaps, and amounting to 0.38 per cent of the serum.

Thus there were found in the serum:

- A. Neutral fat and cholesterol, 14.06 per cent.
- B. Cholesterol (unsaponifiable matter), 1.5 per cent.
- C. Fatty acids originally in the form of soaps, 0.38 per cent.

*Fraction A.* Neutral fat and cholesterol gave the iodine value, by Wijs's method, 78.6, and the saponification value, after deducting cholesterol, 190; the amount of insoluble fatty acids obtained by saponification of this portion was 91.2 per cent of the neutral fat, and these acids then had the iodine value 73.0. The products of saponification soluble in acid obtained from 2 grams of the extract were boiled with nitric acid for some time and examined for phosphoric acid, but no phosphomolybdate of ammonia was formed.

*Fraction B,* containing the cholesterol, was remarkably colourless and crystallized as if it were almost pure cholesterol. A solution of it in alcohol containing 0.2237 of a gram was precipitated with digitonin by Windaus's method, and yielded a precipitate of the cholesteride corresponding to 0.2190 of a gram of free cholesterol; so esters of cholesterol cannot have been present in appreciable amounts. The iodine value of the unsaponifiable matter when first obtained was 118 by Wijs's method; seven months later when determined again by this method it had fallen to 89, but by Hübl's method the theoretical value for pure cholesterol was then given; *viz.*, 65. The melting point of the unsaponifiable

matter without being purified by recrystallization was 141–142°C. Pure cholesterol melts at 145–146°.

*Fraction C*, the fatty acids originally present in the serum as soaps, gave the iodine value 88.6.

Some of the subjacent corpuscular layer was heated with potash and alcohol, as in Liebermann's method of fat estimation; on acidification and shaking with petroleum, fatty acids were obtained amounting to 0.15 per cent, which is not more than is found in normal blood corpuscles.

1. The fat in the blood of this case was present, therefore, in large amount: larger amounts have been recorded; for instance, 18 per cent (Fischer),<sup>2</sup> 19.7 per cent (Neisser and Derlin),<sup>3</sup> 29 per cent (Adler).<sup>4</sup> But it was evident that the amount could vary considerably according to the level in the body of the vessels from which the blood was obtained.

2. The fat was composed almost entirely of simple glycerides and contained but little if any of the phospholipines, such as lecithin. For on saponification the fat yielded 91.2 per cent of insoluble higher fatty acids, whereas lecithin with the conventional formula yields at most 70 per cent, other phospholipines less still, and the simple glycerides of adipose tissue about 95 per cent. The fact that less than this amount was obtained may be explained by supposing that a small amount of some lower soluble acids, such as butyric acid, took part in the composition of the glycerides, and need not indicate the presence of phospholipines, the absence of which was made clear by the failure to detect any phosphoric acid.

3. The fatty acids entering into the composition of the fat, as shown by their iodine value 73, were similar to those obtained from adipose tissue (iodine value about 65) and quite different from those normally found in the liver, heart, or other organs. The liver of this same patient gave 5.7 per cent of fatty acids, by the same treatment and method of estimation, having the iodine value 104 (this fat in the liver was much more conspicuous in the capillaries than in the cells), the heart gave 2.36 per cent, with iodine value 132, and the kidney 3.72 per cent, a large amount for this organ, with iodine value 105. These data point to the cause

<sup>2</sup> B. Fischer: *Virchows Arch. f. path. Anat.*, clxxii, pp. 30 and 218, 1903.

<sup>3</sup> E. Neisser and L. Derlin: *Ztschr. f. klin. Med.*, li, p. 428, 1904.

<sup>4</sup> M. Adler: *Berl. klin. Wchnschr.*, xlv, p. 1453, 1909.

of the lipaemia in this case being the mobilization of connective tissue fat; the other possibility, accumulation in the blood of fat absorbed from the food, is rendered improbable by the large amount, which in the whole blood can hardly have been less than 300 grams and was probably more.

4. The amount of soap in the blood is striking; though in comparison with the amount of neutral fat it is small, absolutely as much fatty acid was present in this form as is often present in all kinds of combination in normal blood.

5. The amount of cholesterol, 1.5 per cent of the serum, rather more than one part for every ten parts of fat in the blood, is also striking; but still larger amounts have been found; *e.g.*, 2.6 and 3.6 per cent (Adler).

6. No evidence of the presence of lecithin in the serum was obtained. The tendency for the amount of lecithin in the blood in diabetic lipaemia to be small has been pointed out by Adler and by Klemperer and Umber.<sup>5</sup>

The above work was carried out under the direction of Prof. J. B. Leathes, to whom I am much indebted for his kind interest and suggestions.

<sup>5</sup> G. Klemperer and H. Umber: *Ztschr. f. klin. Med.*, lxxv, p. 340, 1908.

## FIBRIN.

By A. W. BOSWORTH.

(From the Boston Floating Hospital, Boston, and the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva.)

(Received for publication, November 30, 1914.)

Certain observations made while working with blood seemed to indicate that fibrin might possess some chemical properties quite similar to those of casein, which have been reported by Van Slyke and Bosworth.<sup>1</sup> In order to investigate this question it became necessary to prepare pure ash-free fibrin.

*Method of preparing ash-free fibrin.* Fresh ox blood was collected in a large bottle and carried immediately to the laboratory where it was transferred to precipitating jars and allowed to coagulate. The clots were removed, broken into small pieces, and washed in running water to remove the serum and blood corpuscles. The washed masses of fibrin were passed through a meat chopper, placed in an 8 liter bottle, a little toluene was added, and the bottle filled with a 0.2 per cent solution of sodic hydrate. This solution caused the fibrin to swell, and after about 36 hours the whole contents of the bottle resembled a thin jelly. This jelly was broken up, one-half transferred to another 8 liter bottle, and after the two bottles were filled by the addition of water they were allowed to stand another 36 hours. The jelly was almost completely dissolved, so the contents of the two bottles were filtered, first through cheese cloth, then linen, and finally paper. The clear filtrate was divided into several portions which were placed in 2 liter precipitating jars, diluted with an equal volume of water, and the fibrin was precipitated by the cautious addition of 0.3 per cent acetic acid. At a certain point a flocculent precipitate appeared which quickly settled to the bottom of the jar.

The supernatant liquid was poured off, the precipitate washed with water, dissolved in dilute sodic hydrate (0.05 per cent), and again precipitated with acetic acid. This process was repeated three times, the final precipitate being washed with alcohol and ether and dried over sulphuric acid in an evacuated desiccator. The fibrin strongly resembled casein in all stages of its preparation except in its extreme sensitiveness to a slight excess of acid or alkali, for unlike casein it is readily soluble in weak acetic acid.

<sup>1</sup> This *Journal*, xiv, pp. 203-206, 1913.



The final product was a very fine, light, white powder which gave the following figures upon analysis.

	per cent
Moisture.....	1.84
Ash in dry substance.....	0.03
Carbon in dry substance.....	51.82
Hydrogen in dry substance.....	6.90
Nitrogen in dry substance.....	17.21
Sulphur in dry substance.....	0.95
Oxygen in dry substance*.....	23.12

\*By difference.

In order to show that the preparation obtained was not a substance or mixture of substances resulting from the hydrolysis of fibrin by the alkali used to dissolve the fibrin, the following experiment was performed.

Some of the fibrin, prepared as above, was dissolved in 0.2 per cent sodic hydrate, allowed to stand for 24 hours at 37°C., and the fibrin precipitated by means of acetic acid. The precipitate, after being washed with water, alcohol, and ether, was found to give the same figures upon analysis as the original preparation. The filtrate contained soluble nitrogen which could not be precipitated by acids, showing that hydrolysis had occurred, but that the products of the hydrolysis did not contaminate the final preparation of fibrin.

#### THE RELATION OF FIBRIN TO BASES.<sup>2</sup>

*Combinations with sodium.* One gram of fibrin was found to require 30.7 cc. of  $\frac{N}{50}$  sodium hydroxide to make it neutral to phenolphthalein, or one gram of fibrin combines with  $6.14 \times 10^{-4}$  gram equivalents of sodium to form a compound neutral to phenolphthalein.

One gram of fibrin dissolved in 50 cc. of  $\frac{N}{50}$  sodium hydroxide requires the addition of 42.4 cc. of  $\frac{N}{50}$  HCl to produce the first sign of a precipitate, or one gram of fibrin combines with  $1.52 \times 10^{-4}$  gram equivalents of sodium to form a compound soluble in water.

One gram of fibrin dissolved in 50 cc. of  $\frac{N}{50}$  sodium hydroxide requires the addition of 50 cc. of  $\frac{N}{50}$  HCl to cause the complete precipitation of the fibrin. This proves that fibrin does not form an insoluble salt containing sodium.

<sup>2</sup> For the details of the technique involved in these studies consult the work of Van Slyke and Bosworth upon the caseinates (*loc. cit.*).

*Combinations with calcium.* One gram of fibrin was found to require 30.8 cc. of  $\frac{N}{50}$  calcium hydroxide to make it neutral to phenolphthalein, or 1 gram of fibrin combines with  $6.16 \times 10^{-4}$  gram equivalents of calcium to form a compound neutral to phenolphthalein.

One gram of fibrin dissolved in 100 cc. of  $\frac{N}{50}$  calcium hydroxide requires the addition of 77.5 cc. of  $\frac{N}{50}$  HCl to produce the first sign of a precipitate, or one gram of fibrin combines with  $4.50 \times 10^{-4}$  gram equivalents of calcium to form a compound soluble in water.

One gram of fibrin dissolved in 100 cc. of  $\frac{N}{50}$  calcium hydroxide requires the addition of 70.0 cc. of  $\frac{N}{50}$  HCl to cause a complete precipitation of the fibrin, or one gram of fibrin combines with  $3.0 \times 10^{-4}$  gram equivalents of calcium to form a compound insoluble in water. This precipitate is completely dissolved by 5 per cent solution of sodium chloride.<sup>3</sup>

*Relation of fibrin to hydrochloric acid.*

One gram of fibrin dissolved in 50 cc. of  $\frac{N}{50}$  HCl requires the addition of 42.5 cc. of  $\frac{N}{50}$  sodium hydroxide to produce the first sign of a precipitate, or one gram of fibrin combines with  $1.50 \times 10^{-4}$  gram equivalents of hydrochloric acid to form a compound insoluble in water.

*Effect of carbon dioxide on solutions of fibrinates.*

Fibrin is not precipitated by running a stream of carbon dioxide into solutions of sodium, potassium, or ammonium fibrinates, while solutions of calcium or barium fibrinates give precipitates of acid fibrinates. Solutions of calcium fibrinate clot upon being exposed to the air, due to the absorption of carbon dioxide.

Fibrin, unlike casein, does not decompose calcium carbonate when these two substances are triturated together in the presence of water.

<sup>3</sup> Casein forms a similar compound with calcium (this *Journal*, xiv p. 231, 1913).

*Molecular weight of fibrin.*

From the sulphur content we have,

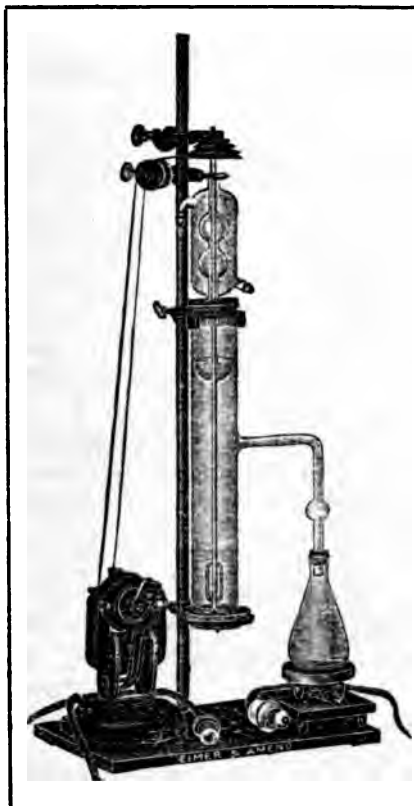
$$\text{Mol. Wt.} = N \left[ \frac{32.07}{0.95} \times 100 \right] = N \ 3375.7. \quad \text{If } N = 2, \text{ then Mol. Wt.} = 6751.4.$$

From the sodium fibrinate containing one equivalent of base we have,

$$\text{Mol. Wt.} = \frac{1}{1.5 \times 10^{-4}} = 6666.6.$$

## CONCLUSIONS.

1. Fibrin can combine with both bases and acids to form definite compounds.
2. Fibrin combines with four equivalents of base to form a compound which is neutral to phenolphthalein.
3. Fibrin combines with bases to form a series of three acid salts which contain one, two, and three equivalents of base respectively.
4. All the combinations of fibrin with sodium, potassium, and ammonium are soluble.
5. The calcium fibrinates containing three and four equivalents of calcium are soluble, the calcium fibrinates containing one and two equivalents of calcium being insoluble.
6. Fibrin combined with one equivalent of acid is insoluble, and combined with more than one equivalent of acid is soluble.
7. Pure fibrin unlike casein is not strong enough as an acid to decompose calcium carbonate.
8. The molecular weight of fibrin is about 6666.
9. Carbon dioxide precipitates fibrin from a solution of calcium fibrinate, but not from a solution of sodium, potassium, or ammonium fibrinate.



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**AN IMPROVED METHOD FOR THE ESTIMATION OF  
INORGANIC PHOSPHORIC ACID IN CERTAIN  
TISSUES AND FOOD PRODUCTS.<sup>1</sup>**

By ROBERT M. CHAPIN AND WILMER C. POWICK.

(From the Biochemic Division, Bureau of Animal Industry, U. S. Department  
of Agriculture, Washington.)

(Received for publication, December 12, 1914.)

INTRODUCTION.

For the solution of the much discussed questions as to the precise significance of the organically and of the inorganically combined phosphorus in animal and in vegetable tissues, a method for sharply and surely differentiating between these two classes of compounds and for estimating accurately the amount of phosphorus combined in each is obviously essential. With a view to supplying this need, many methods, differing among themselves in accuracy and in practicability, and each more or less specifically adapted to the investigation of a certain limited range of materials, have been evolved; but as yet no method has been found, if indeed one ever can be found, that is universally superior in its application to all substances. The improved method here offered, however,—designed for and successfully used in the determination of inorganic phosphorus in eggs and in meats,—is believed to possess a sufficient number of points of superiority over its immediate competitors, together with a sufficiently wide range of applicability, to justify its publication at this time.

DESCRIPTIVE PART.

*Discussion of improvements claimed for the method.*

Of the methods now available for the determination of inorganic phosphorus in flesh, there are three which seem to stand pre-

<sup>1</sup> Published by permission of the Secretary of Agriculture.



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eminent; *viz.*, the Emmett and Grindley method,<sup>2</sup> the Siegfried and Singewald method,<sup>3</sup> and the Forbes method<sup>4</sup>—each of which has undoubtedly proved reasonably satisfactory in its particular field. Yet their failure to guard sufficiently well against tedious filtrations, decomposition of organic phosphorus compounds, and incomplete separation of the organic from the inorganic phosphorus, makes problematical the accuracy and practicability of each when applied to tissues other than flesh, or even to that tissue after the occurrence of degenerative changes.

The requirements of a generally trustworthy method for the determination of inorganic phosphorus in physiological tissues, the shortcomings of the above mentioned methods, and the advantages claimed for the improved method are briefly summarized below.

*Extraction.* To insure the solution of any water-insoluble phosphates that might be present, the extracting medium should be acid. To avoid any possible chemical decomposition of the organically combined phosphorus, the use of strong reagents and of heat should be avoided. As a precaution against bacterial and enzymatic changes, the extraction should be effected quickly, in the cold, and in the presence of an antiseptic. Most simply to prevent the interference of colloids in the subsequent phosphate precipitation and the unnecessary exposure of the organic phosphorus compounds to the action of the precipitant, these substances should be rendered initially insoluble in the extracting medium, either by physical or by chemical methods. For obvious reasons, time-consuming operations should be avoided as far as possible.

The Emmett and Grindley method falls short of the above requirements by the use of a neutral solvent and of heat; the Siegfried and Singewald method, by failure to remove the protective colloids, by exposure of the organic phosphorus to the action of the phosphate precipitant, and by the long duration of the initial

<sup>2</sup> A. D. Emmett and H. S. Grindley: *Jour. Am. Chem. Soc.*, xxviii, p. 25, 1906; H. S. Grindley and E. L. Ross: *this Journal*, viii, p. 483, 1910.

<sup>3</sup> M. Siegfried and E. Singewald: *Ztschr. f. Untersuch. d. Nahrungs u. Genussmittel*, x, p. 521, 1905.

<sup>4</sup> E. B. Forbes, A. Lehmann, R. C. Collison, and A. C. Whittier: *Bull. 215, Ohio Agric. Exper. Sta.*, 1910.

filtration; and the Forbes method, by the use of heat and the necessity for a double filtration. Finally, Collison's<sup>5</sup> modification of the Forbes method of extraction is open to the criticism that the proteid superficially coagulated by the strong alcohol interferes with the further penetration of the tissue by the solvent.

In the method herein proposed, however, the above requirements have been met by employing as the extracting medium an aqueous solution of picric acid containing a small amount of hydrochloric acid. The extraction is complete; bacterial action is prevented and the proteids are effectually coagulated by the reagent; while the danger of chemical or enzymatic changes is minimized by the low temperature and the rapidity of the extraction. The extract is easily filtered and is practically free from organically combined phosphorus; while by the use of an aliquot of the filtrate for further work, a tedious washing of the precipitate is avoided.

*Separation of the organic from the inorganic phosphorus.* In view of the meagerness of our knowledge regarding the organic phosphorus compounds in physiological tissues, the separation of these from the inorganic phosphates must be conducted largely according to *a priori* considerations. Upon such grounds it would appear that the best assurance of a quantitative separation of the inorganic from the organic phosphorus and from other contaminating substances is to be found in an at least partial separation at the time of the extraction (which we have secured by the use of picric acid) and a subsequent double precipitation of the inorganic phosphates, once from acid and once from alkaline solution. Obviously, the precipitant first to be employed should be that which is least likely to decompose the organic phosphorus compounds, or, by precipitating them, to expose them to decomposition in subsequent operations; while the final precipitant should be that which yields the precipitate best adapted for the estimation of small amounts of phosphoric acid.

Since, in the initial use of ammonium molybdate,—the only available acid precipitant,—the presence of strong reagents and the precipitation of more or less of the organic matter are involved, and since in its use as a final precipitant a comparatively heavy precipitate is obtained from a relatively small amount

<sup>5</sup> R. C. Collison: *Jour. Ind. and Eng. Chem.*, iv, p. 606, 1912.

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of phosphoric acid, the initial use of an alkaline precipitant is clearly indicated. Of these, magnesia mixture seems to be preferable to either barium chloride or calcium chloride, for by its use a clear filtrate is easily obtained and the use of sulphuric acid in subsequent operations is not rendered impossible.

In the method here proposed, therefore, the initial precipitation has been made by means of magnesia mixture and the final precipitation by means of ammonium molybdate, which, we believe, insures for the method a further superiority over the Emmett and Grindley method, in which the order is reversed.

*Final estimation of the inorganic phosphoric acid.* Unquestionably the inorganic phosphorus should be determined directly and not obtained by difference; for it is the form of phosphorus concerning which the most precise information is usually desired, and it is likewise the form which is most amenable to exact determination.

There has been much study, resulting in a voluminous literature, upon the final estimation of phosphoric acid; but as a criticism or comparison of the methods from time to time proposed does not lie within the scope of this paper, the reader is referred to the work of Artmann,<sup>6</sup> which contains a critical review of the methods now available. The present writers have chosen to make their final estimation gravimetrically, by the direct weighing of the ammonium phosphomolybdate: first, because the previous procedure in their method leads most logically (as has already been pointed out) to this method of final estimation; and secondly, which is more important, because the precipitate of ammonium phosphomolybdate is much larger (nineteen times heavier) than a chemically equivalent precipitate of magnesium pyrophosphate, an important consideration in view of the small amount of phosphoric acid usually dealt with.

The particular method of estimation that we have chosen is that of Lorenz,<sup>7</sup> which has been used with satisfaction by a number of workers, including especially Neubauer and Lücker,<sup>8</sup> who have proved its reliability and have suggested slight modifications in its technique in the interests of convenience and economy.

<sup>6</sup> P. Artmann: *Ztschr. Angew. Chem.*, xxvi, pt. I, p. 203, 1913.

<sup>7</sup> N. V. Lorenz: *Die landwirtschaftl. Versuchsst.*, lv, p. 183, 1901; *Österr. chem. Ztg.*, xiv, p. 1, 1911.

<sup>8</sup> H. Neubauer and F. Lücker: *Ztschr. f. anal. Chem.*, li, p. 161, 1912.

*Detailed execution of the method.*

*Extraction.* Rejecting the method of extracting the phosphoric acid by exhausting the tissue by repeated washings, we have chosen, in the interests of convenience and simplicity and in order to secure a more concentrated extract, to subject the material to a single, thorough maceration with the solvent, and to employ an aliquot of the filtrate for further work. We have devised three methods by which to determine the exact concentration of the extract in terms of the original material, thereby avoiding errors due to the presence of an originally unknown volume of water or of insoluble matter in the sample. These methods are described below as Modifications A, B, and C.

*Modification A.* This modification is applicable when the water content of the sample is known or can be determined with sufficient accuracy.

A weighed sample of the material, containing between 8 and 80 mgm. of inorganic  $P_2O_5$ , is macerated in a porcelain mortar with 20 grams of dry, acid-washed sand, and is thoroughly, but quickly, mixed with an accurately measured amount of water (about 200 cc.) and 10 cc. of 2.5 normal hydrochloric acid. The mixture is transferred to a wide mouthed, glass-stoppered bottle of about 500 cc. capacity, and 5 to 8 grams of powdered picric acid are added. The bottle is shaken at frequent intervals during the next two hours, or continuously, by machine, for one-half hour; after which the extract is filtered through a folded filter, and 100 cc. of the filtrate are measured out for subsequent work.

The volume of the whole solution is found by adding to the volume of water and hydrochloric acid employed the volume of water contained in the sample; and the proportional amount of the original sample represented by 100 cc. of the filtrate is then calculated.

*Modification B.* This modification is applicable when the volume of the insoluble matter is known or is negligible.

The weighed material is ground with sand in a mortar, as under Modification A. The whole is then diluted and quantitatively transferred with water to a graduated flask or stoppered cylinder, where it is treated with 10 cc. of 2.5 normal hydrochloric acid and 5 to 8 grams of powdered picric acid as before. It is then

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diluted with water to a definite total volume, and shaken, filtered, and sampled, as described under Modification A.

To determine the volume displaced by the insoluble matter, the residue is sucked dry, its weight and specific gravity are determined, and its volume is calculated. If the use of sand is unnecessary and the amount of insoluble matter is negligible, obviously the volume correction may be omitted; while if sand is employed, but the volume of the insoluble matter is negligible, only a once for all determined correction for the volume of the sand need be applied, provided, however, that in each case the picric acid is added after the apparent volume of the solution has been observed.

*Modification C.* In this case a "marker" is used to indicate the concentration of the resultant extract, on the principle that if a definite amount of some inert chemical that is not naturally present in the material under examination be added at the beginning, a determination of the concentration of such chemical in the final extract will afford a measure of the total volume of liquid in which this chemical, and therefore the  $P_2O_5$ , is dissolved. A half normal solution of potassium iodide has been chosen for this purpose, and the urea-nitrite method of Schirmer<sup>9</sup> has been used for the determination of the potassium iodide in the filtered extract and for the standardization of the half normal potassium iodide solution. The method of Kendall,<sup>10</sup> which was first tried, proved to be inapplicable in the presence of picric acid, while the other methods of Schirmer were less suited to our purpose than the method chosen.

In this modification the weighed material is ground with sand as under Modification A, and by the aid of water is quantitatively washed into a 500 cc. glass-stoppered bottle and brought to a volume of about 200 cc. The mixture is then treated successively with 10 cc. of 2.5 normal hydrochloric acid, exactly 25 cc. of the half normal potassium iodide solution, and from 5 to 8 grams of powdered picric acid. The bottle is shaken and its contents filtered, as described under Modification A; and from the filtrate two portions of 50 cc. and 100 cc. are measured for the potassium iodide and for the phosphoric acid determinations, respectively.

<sup>9</sup> W. Schirmer: *Arch. d. Pharm.*, ccl, p. 448, 1912.

<sup>10</sup> E. C. Kendall: *Jour. Am. Chem. Soc.*, xxxiv, p. 894, 1912.

The 50 cc. portion is measured into a spacious, narrow necked, glass-stoppered bottle, treated with 10 cc. of five normal sulphuric acid and 15 cc. of a freshly prepared 1 per cent sodium nitrite solution, and after one to two minutes with 1.5 grams of crystallized urea. The solution is shaken vigorously in the tightly stoppered bottle until the excess of nitrous acid is destroyed, after which the separated iodine is dissolved by the addition of 10 cc. of normal potassium iodide solution and titrated in the usual manner against thiosulphate in the presence of starch. The thiosulphate equivalent of 5 cc. of the original half normal potassium iodide solution is determined in the same manner, and multiplied by five to obtain the thiosulphate equivalent of the potassium iodide originally added to the sample. If this figure be represented by A cc., and the thiosulphate required for the 50 cc. aliquot of the filtrate by B cc., and the weight of the tissue operated upon by W gm., then the 100 cc. of the filtrate to be used for the  $P_2O_5$  determination will obviously represent  $\frac{2BW}{A}$  grams of the original material.

*Separation of the inorganic phosphoric acid.* The phosphoric acid in the 100 cc. aliquot of the filtrate obtained by either of the above modifications of the extraction process is precipitated in the usual manner with magnesia mixture and ammonia; and the precipitation is completed, or allowed to complete itself, by agitation or by sufficient standing. The precipitate is filtered off and washed with 2.5 per cent ammonia water until the washings are practically colorless; then the precipitate on the filter and in the beaker is dissolved in dilute nitric acid, and washed with water into a 150 cc. beaker. The combined solution and washings are then evaporated to dryness and the residue is redissolved in a mixture of 25 cc. of nitric acid (sp. gr., 1.20), 1 cc. of concentrated sulphuric acid, and 25 cc. of water.<sup>11</sup>

*Final estimation of the inorganic phosphoric acid.* The final estimation of the inorganic phosphoric acid in the nitric-sulphuric acid solution of the dry residue, prepared as above, we have carried out by the Lorenz method, with exact adherence to the directions which he has given, both for the precipitation

<sup>11</sup> Ammoniacal alcohol might, of course, be used for the washing of the magnesium ammonium phosphate precipitate and acidified alcohol for dissolving it, according to the method of Forbes; but this procedure has not appeared to be necessary in our work thus far.

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and for the filtration and washing, except that in some cases we have used acetone for the washing, as suggested by Neubauer and Lückner, in place of alcohol and ether, as directed by Lorenz. The use of acetone for this purpose we have found to be perfectly satisfactory, provided only that the acetone is free from aldehydes and is otherwise pure.

Discussion and explanation of the various steps of this method are to be found in the original references, and for the sake of brevity will be omitted here.

### EXPERIMENTAL PART.

#### *Experimental results in the development and application of the improved method.*

*Lorenz method.* Chemically pure sodium biphosphate was dissolved in water, and the solution was standardized by weighing both the residue obtained by the evaporation of 100 cc. in a platinum dish, and the residue of sodium metaphosphate subsequently obtained on ignition. Duplicate determinations all led to identical values for the concentration of the solution.

The  $P_2O_5$  factor of the precipitate yielded by the Lorenz method was determined by weighing the precipitates obtained from varying quantities of the above solution, conditions in duplicate determinations being purposely varied between the limits specified by Lorenz without appreciable irregularities resulting. The method was found to be thoroughly satisfactory, and the  $P_2O_5$  factors determined by us differed from those given by Lorenz by no more than the experimental error, as shown in the following table:

TABLE 1.  
 *$P_2O_5$  factors for use with the Lorenz method.*

APPROXIMATE WEIGHT OF PRECIPITATE	FACTOR	
	Found by us	Given by Lorenz
<i>gm.</i>		
1.5	0.03307	0.03291
1.0	0.03305	0.03295
0.8	0.03298	0.03299
0.4	0.03312	
0.3		0.03301
0.12	0.03322	0.03307

Originally the factors obtained by us for small amounts of  $P_2O_5$  were appreciably lower than those shown in the table, but this was found to be due to a separation of  $MoO_3$  that had taken place in consequence of an insufficient amount of nitric acid in the reagent. In such work as was done with this reagent, however, the factors experimentally found with that solution were used. Lorenz has recommended the general use of the factor 0.03295, which, being based upon more exhaustive work than that done by us, we are inclined to accept in preference to our slightly different figures, particularly as the difference may be due to our having used a less pure salt.

TABLE 2.  
*Recovery of  $P_2O_5$  from picric acid solution.*

	1	2	3	4
$P_2O_5$ used, gm.....	0.004106	0.004106	0.004106	0.006159
Weight of precipitate, gm.....	0.1257	0.1265	0.1257	0.1888
Factor.....	0.03259	0.03259	0.03259	0.03259
$P_2O_5$ recovered, gm.....	0.004097	0.004113	0.004097	0.006153
$P_2O_5$ recovered, per cent.....	99.77	100.11	99.77	99.90
$P_2O_5$ recovered, average per cent.....				99.89

*Effect of picric acid on the recovery of  $P_2O_5$ .* Measured portions of the standard phosphate solution were diluted to 100 cc. with a saturated solution of picric acid, and precipitated with magnesia mixture and ammonia. After twelve hours the precipitates were filtered off and washed, dissolved in dilute nitric acid, and the phosphoric acid was determined by the method already indicated. The results are given in Table 2.

Considering the small volume of the standard phosphate solution that was used, the results shown in Table 2 are extremely satisfactory.

*Recovery of  $P_2O_5$  added to eggs and to meat.* In the next set of experiments a considerable quantity of whole egg substance was thoroughly mixed, and its water content was determined by drying a sample in a vacuum oven at about 65°C. Inorganic phosphoric acid determinations were then made upon portions of the original egg substance and likewise upon portions of the egg



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substance to which known amounts of the standard phosphate solution had been added, with a view to ascertaining how much of the added phosphoric acid could be recovered. The determinations were carried out by the method already described, the picric acid extract being made by Modification A. The results of this experiment are given in Table 3.

In another similar experiment on meat (see Table 6) in which the extracts were made by Modification C, 98.73 per cent, 101.12 per cent, and 98.97 per cent were recovered in triplicate determinations, making an average recovery of 99.61 per cent.

The difference between the amounts of phosphoric acid added and the amount recovered in the above experiments is in each case small; and since the loss or gain is divided between two separate determinations, each of which, in the case of the experiment on meat, involves an iodine as well as a phosphoric acid determination, the errors appear to be well within the permissible limits.

*Experiments on the iodine method for ascertaining the concentration of the extract.* The first step in this series of experiments was to check the Schirmer method for the estimation of potassium iodide.

A standard solution of potassium iodide was prepared from a weighed amount of the pure substance, and was checked by titration against thiosulphate by Kendall's method. The standard solution of thiosulphate had been based upon standard dichromate in the usual way. It was calculated that 1 cc. of standard potassium iodide solution should be equivalent to 0.3288 cc. of the standard thiosulphate. The results of the tests on Schirmer's method, both in the presence and in the absence of picric acid, are given in Table 4.

The Schirmer method having been shown to be satisfactorily accurate, the whole method of  $P_2O_5$  determination according to Modification C was then carried out on a standard phosphate solution as follows:

To 10 cc. of a standard phosphate solution, containing a total of 0.0154 of a gram of  $P_2O_5$ , were added 25 cc. of a standard potassium iodide solution. To this mixture were added 10 cc. of a 2.5 normal hydrochloric acid solution, 5 grams of picric acid, and sufficient water to make the volume about 250 cc. Of the final solution two aliquot portions of 25 cc. were used for the

TABLE 3.  
*Recovery of a known amount of  $P_2O_5$  added to eggs.*

	A			B		
	NO ADDITION			NO ADDITION		
	1	2	3	1	2	3
Gm. $P_2O_5$ per cc. standard solution.....						
Cc. added.....	0.00	0.00	0.00	0.00	0.00	0.002046 4.989
Weight of added $P_2O_5$ .....	0.00	0.00	0.00	0.00	0.00	0.010207 0.010207
Weight of egg substance, gm.....	40.00	40.00	40.00	40.00	40.00	40.00
Per cent of moisture.....	73.25	73.25	73.25	74.34	74.34	74.34
Volume of water in charge, cc.....	29.3	29.3	29.3	29.74	29.74	29.74
Volume of water + $P_2O_5$ solution, cc.....	144.2	144.2	144.2	150.09	150.09	155.08
Volume 2.5 N HCl.....	10.0	10.0	10.0	10.0	10.0	10.0
Total volume of solution.....	183.5	183.5	183.5	189.83	189.83	194.82
Weight of precipitate from 100 cc.....	0.1362	0.1384	0.1361	0.1162	0.1168	0.2750
$P_2O_5$ factor.....	0.03259	0.03259	0.03259	0.03259	0.03259	0.032834
Weight of $P_2O_5$ in 100 cc.....	0.004439	0.004435	0.004435	0.003787	0.003807	0.009013
Weight of $P_2O_5$ in whole charge.....	0.008145	0.007978	0.008139	0.007189	0.007226	0.017591
Average $P_2O_5$ in egg used.....			0.008087		0.007207	0.007207
Difference: added $P_2O_5$ recovered.....			0.010093			0.010384
Per cent of added $P_2O_5$ recovered.....			98.32			101.73
Average per cent of recovery.....			98.15			101.57

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TABLE 4.

*Results on standard potassium iodide obtained with Schirmer's urea-nitrite method.*

	WITHOUT PICRIC ACID			WITH PICRIC ACID		
KI solution used, cc.....	25.00	25.00	25.00	25.00	25.00	25.00
Thiosulphate required, cc.....	8.20	8.24	8.20	8.24	8.30	8.21
Average titration.....			8.24			8.25
Calculated titration.....			8.22			8.22

iodine determination, and two portions of 50 cc. for the phosphoric acid determination by the combined magnesia mixture and Lorenz methods. The results are given in Table 5.

It remained finally to check the accuracy of Modification C in the presence of proteid, for which purpose the following experiment was carried out.

From a thoroughly ground sample of beef round, six portions of about 20 gm. each were accurately weighed into large porcelain mortars containing about 20 gm. of acid-washed sand. These samples were then each ground, with the addition of a little water, to a smooth paste, transferred quantitatively, with the aid of about 200 cc. of water, to a glass-stoppered bottle of about 500 cc. capacity, and treated successively with 10 cc. of 2.5 normal hydrochloric acid, 5 gm. of picric acid, and 25 cc. of half normal

TABLE 5.

*Application of the whole method (Modification C) to standard  $P_2O_5$  solution.*

	1	2
Thiosulphate required for whole amount of KI used.....	96.50 cc.	96.50 cc.
Thiosulphate required for 25 cc. aliquot.....	9.75 cc.	9.74 cc.
Average.....		9.745 cc.
Per cent of whole solution represented by 25 cc.		10.098
Per cent of whole solution represented by 50 cc.		20.196
Weight of precipitate from 50 cc.....	0.0962 gm.	0.0978 gm.
Weight of $P_2O_5$ recovered from 50 cc. (average).		0.003162 gm.
Weight of $P_2O_5$ recovered from whole solution ( $\frac{0.0978}{0.20196}$ ).....		0.01566 gm.
Weight of $P_2O_5$ added.....		0.01564 gm.
Per cent of added $P_2O_5$ recovered.....		100.11

potassium iodide solution. To three of the samples, 25 cc. portions of a solution containing 0.001564 gm. of  $P_2O_5$  per cc. were added, and the inorganic phosphoric acid in each sample was then determined by Modification C of the method that we have already described. The results of this experiment are shown in Table 6.

TABLE 6.

*Application of Modification C to recovery of phosphoric acid added to beef.*

	WITHOUT $P_2O_5$ ADDITION			WITH $P_2O_5$ ADDITION		
	1	2	3	1	2	3
KI solution used, cc.....	25.00	25.00	25.00	25.00	25.00	25.00
Thiosulphate equivalent of above	110.80	110.80	110.80	110.80	110.80	110.80
Volume of extract used for iodine determination.....	50.00	50.00	50.00	50.00	50.00	50.00
Thiosulphate required for 50 cc....	19.96	20.08	20.35	19.70	20.21	22.96
Part of whole represented by 50 cc.....	0.18014	0.18123	0.18366	0.1778	0.1824	0.20722
Part of whole solution represented by 100 cc.....	0.36028	0.36246	0.36732	0.3556	0.3648	0.41444
Charge.....	20.1618	20.0316	20.0445	19.9606	20.0095	20.3118
Gm. of meat represented by 100 cc. aliquot.....	7.2639	7.2607	7.3627	7.0980	7.2995	8.4180
Weight of phosphomolybdate precipitate.....	0.6253	0.6210	0.6421	1.0270	1.0654	1.2097
$P_2O_5$ factor.....	0.03301	0.03301	0.03301	0.03304	0.03304	0.03307
Weight of $P_2O_5$ in 100 cc. (total)...	0.02064	0.02050	0.02119	0.03394	0.03521	0.04000
Per cent of "natural" $P_2O_5$ in meat.....	0.2841	0.2823	0.2878			
Average of first set.....			0.2847			
Weight of "natural" $P_2O_5$ in 100 cc.....				0.02021	0.02078	0.02396
Weight of added $P_2O_5$ determined in 100 cc.....				0.01373	0.01442	0.01604
$P_2O_5$ originally added.....				0.03910	0.03910	0.03910
Added $P_2O_5$ recovered (calculated for whole).....				0.03861	0.03954	0.03870
Per cent of added $P_2O_5$ recovered.....				98.73	101.12	98.97
Average percentage of recovery.....						99.61

While the averages of the individual experiments described above, both in the control and in the experiment on meat, show the practically quantitative recovery of the entire amount of phosphoric acid added, yet the recovery was not so nearly quantitative in the individual determinations, which show errors amounting to over 1 per cent of the total phosphoric acid determined. When it is considered, however, that the actual experi-

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mental error arising from a combination of four separate analytical operations is naturally considerable, and that in these experiments such an error is multiplied by three, a final error of  $\pm 1$  per cent does not seem inordinately large.

It may therefore be said that, in the absence of interfering substances, the above described modification leads to rapid and sufficiently accurate results, and for the most part seems to be a very desirable variation. Yet it is felt that the modification has not yet received a sufficiently broad test, and that it leaves something to be desired on account of the possibly limited range of its utility. Thus, it was found that it could not be used upon cold water extracts of meat which had been preserved with thymol, on account of the interfering action of that substance, unless the thymol had been previously expelled.

### *Comparison of the improved method with other well known methods.*

Although the data already obtained had convinced us of the accuracy of our method, it was nevertheless decided to compare it with a few other well known methods, even though in the event of their yielding discrepant results, no conclusion could be formed as to which method might be at fault. The methods selected for this purpose were the Emmett-Grindley, the Siegfried-Singewald, and the Forbes methods. These methods as outlined by Grindley and Ross<sup>12</sup> were followed in all essential respects, except that after the initial phosphomolybdate precipitates had been formed according to directions, the subsequent procedure in all the methods was identical and consisted of a magnesia mixture precipitation followed by a final estimation of the phosphoric acid by the Lorenz method.

The material selected as sample was a cold water extract of flesh, prepared with thymol as a preservative; and for the purpose of comparing the methods, the two following experiments were made.

*Experiment A.* Two liters of a cold water extract were prepared from practically fresh, finely ground beef round and water saturated with thymol. Triplicate determinations were made by each of the three methods

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<sup>12</sup> Grindley and Ross: *loc. cit.*

mentioned, and by the picric acid method, 200 cc. of the extract being used for the picric acid treatment, and 100 cc. for each of the other methods. The total volume of the solution in the picric acid method was determined, after expelling the thymol, by Modification C. The following results were obtained:

METHOD	PER CENT OF SOLUBLE INORGANIC $P_2O_5$ IN ORIGINAL MEAT			
	Results of separate determinations			Average
Forbes magnesia mixture.....	0.2856	0.2848	0.2838	0.2848
Emmett-Grindley.....	0.2792	0.3224*	0.2794	0.2793
Siegfried-Singewald.....	0.2812	0.2800	0.2806	0.2806
Picric acid.....	0.2750	0.2746	0.2740	0.2745

\* Rejected.

*Experiment B.* The previous experiment was repeated, except that the meat used for the preparation of the extract was somewhat older, and that Modification B instead of Modification C was used for the picric acid method. In using Modification B the picric acid was added after the solution had been brought to definite volume, and no correction for volume was then found to be necessary. The results obtained in this experiment are given below:

METHOD	PER CENT OF SOLUBLE INORGANIC $P_2O_5$ IN ORIGINAL MEAT			
	Results of separate determinations			Average
Forbes magnesia mixture.....	0.3519	0.3533	0.3523	0.3525
Emmett-Grindley.....	0.3531	0.3536	0.3546	0.3538
Siegfried-Singewald.....	0.3506	0.3522	0.3487	0.3505
Picric acid.....	0.3463	0.3459	0.3471	0.3464

In each of the above experiments lower results were obtained by the picric acid method than by any of the other methods used. It is believed, however, that the results of the determinations by our method are quantitative, since the method is theoretically correct and has been indicated to be accurate by the experiments already recorded and by numerous others which we have not brought forward. The larger results obtained by the other methods are to be explained, we believe, by the precipitation of some of the organically combined phosphorus, or by the liberation of inorganic phosphorus from organic phosphorus compounds, or both.

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### *The distribution of phosphorus in eggs.*

A few hitherto unpublished results obtained by the use of the picric acid method on eggs are here appended. It was our object in these experiments to determine the ratio between the inorganic and the total phosphorus in different grades of eggs, in order to see if there might exist any relation between this ratio and the degree of deterioration as judged by candling. The experiments showed a progressive increase in this ratio as deterioration advanced. The method used for the determination of the inorganic phosphoric acid was the above proposed picric acid method, Modification A being employed. The total phosphoric acid was determined by igniting the dried egg substance with soda and saltpeter, and, after dissolving the residue and filtering, by precipitating by the Lorenz method. The results obtained are shown in Table 7.

### CONCLUSION.

1. An improved method for the determination of inorganic phosphoric acid in tissues has been devised, which, on *a priori* grounds, should yield correct results, and which has the following advantages over the methods customarily used:

a. It does not involve the action of heat or of strong reagents upon the organic matter present in the tissue.

b. Bacterial decomposition of the organic matter is prevented by the presence of picric acid, a powerful antiseptic.

c. The precipitation is effected after the removal of the proteid matter, and therefore is not influenced by the protective action of colloids; while contamination with organic compounds of phosphorus is reduced to a minimum.

d. Tedious filtrations are avoided by using an aliquot portion of the filtrate from the picric acid coagulum for the determination of inorganic phosphoric acid.

e. A rapid and accurate chemical method has been employed for determining the proportional part of the whole solution represented by the aliquot part used.

2. The method described, and its several modifications, have been experimentally tested and found to be rapid and accurate.

TABLE 7.

*Distribution of phosphorus in different grades of eggs.*

DESCRIPTION OF SAMPLE	LABORATORY NUMBER	MOISTURE	INORGANIC $P_2O_5$ IN		TOTAL $P_2O_5$ IN		PER CENT OF TOTAL $P_2O_5$ IN INORGANIC STATE
			Fresh egg	Dry substance	Fresh egg	Dry substance	
Fresh eggs, 24 hrs. old	49		0.0165	0.0633			
			0.0159	0.0610			
			0.0159	0.0612			
		73.98	0.0161	0.0618			
Fresh eggs, 24 hrs. old	50		0.0179	0.0689			
			0.0179	0.0686			
			0.0183	0.0704			
			0.0182	0.0703			
			0.0183	0.0704	0.494	1.900	
		73.98	0.0181	0.0697	0.494	1.900	3.670
Fresh eggs, 24 hrs. old	55	74.37	0.0179	0.0700	0.493	1.922	
		74.32	0.0180	0.0703	0.496	1.935	
		74.34	0.0180	0.0701	0.495	1.929	3.555
"Fresh" eggs, as judged by candling test	52	73.71			0.510	1.939	
		73.67	0.0156	0.0592	0.507	1.928	
		73.69	0.0156	0.0592	0.509	1.933	3.064
Second grade eggs, kept frozen for 185 dys.	57	72.72	0.0227	0.0830	0.543	1.988	
		72.67	0.0229	0.0838	0.546	2.002	
		72.70	0.0228	0.0834	0.545	1.995	4.151
Poorer grade of No. 2 eggs kept frozen for 187 dys.	58	69.67	0.0207	0.0687	0.490	1.607	
		69.38	0.0209	0.0679	0.492	1.615	
		69.52	0.0208	0.0683	0.491	1.611	4.239
"Borderline," almost inedible eggs, kept frozen for 187 dys.	59	74.02	0.0279	0.1062	0.627	2.392	
		73.52	0.0279	0.1063	0.619	2.360	
		73.77	0.0279	0.1063	0.623	2.376	4.437
Shell eggs, containing dead, 2 dy. old embryo; in incubator two wks.	54	70.67	0.0408		0.550	1.879	
		70.72	0.0418		0.553	1.886	
		70.70	0.0413	0.1409	0.552	1.882	7.486
"Rots," eggs in a well advanced stage of decomposition	23		0.1294	0.4163	0.536	1.725	
		68.93	0.1280	0.4119	0.546	1.758	
		68.93	0.1287	0.4141	0.541	1.741	23.78



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3. Experiments for the comparison of the picric acid method with the Forbes, the Emmett-Grindley, and the Siegfried-Singewald methods have shown that approximately the same results are obtained by each, when employed for the determination of inorganic phosphorus in the cold water extract of flesh. The slightly lower results obtained by the picric acid method are attributed to the sharper separation of the inorganic from the organic phosphorus compounds that can be obtained by its use.

4. By means of this method, a progressive increase in the ratio of the inorganic to the total phosphorus in eggs has been found, which increase corresponds to the increased deterioration of the eggs as judged by physical means.

## ON THE SIZE AND COMPOSITION OF THE THYMUS GLAND.

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There is abundant evidence to the effect that the thymus is most active during the growth period before puberty, and it is generally assumed that the gland becomes inactive and gradually atrophies after puberty. It would, therefore, be logical to use the glands from healthy, young, growing animals for medicinal purposes, and in the Armour Laboratory these glands are obtained exclusively from suckling calves.

For the purpose of obtaining, if possible, some definite and conclusive information regarding the relative size, physiological activity, and chemical composition of the thymus during intra-uterine life as well as before and after puberty, this investigation was carried out on glands from beef fetuses six to nine months old, from calves two to four months old, and from full grown cattle. Glands from adult hogs were also collected for comparative purposes. Sheep glands were obtained indiscriminately, from young and from full grown animals, and the entire lot represents, therefore, all ages of this species with the exception of newborn lambs and sucklings. It was impossible to secure glands from hog and sheep fetuses and from suckling pigs and lambs in sufficient quantity to be of much, if any, value.

The glands were collected every Tuesday over a period of ten weeks, during June, July, and August, 1914. This time was selected because it is at the height of the outdoor season, and the animals are allowed, at least to a large extent, to choose the quality and quantity of their individual food. In the winter more or less artificial feeding conditions prevail.

The glands were removed from the various animals immediately after slaughter, while still retaining the animal heat, carefully

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trimmed free from connective and other adherent tissues, weighed, and stored at freezing temperature until the entire lot was collected. They were then finely minced, well mixed, and average portions of about 2000 grams dried on enamelled trays to constant weight at a temperature not exceeding 50°C. The dried material was coarsely ground and extracted with petroleum ether in Soxhlet extractors; and the desiccated fat-free material was powdered in a steel tube mill to pass a 60 mesh sieve.

In the tabulation will be found the total number of glands employed, the maximum, minimum, and average weights of the fresh glands together with the moisture, petroleum ether-soluble substance, and yield of desiccated fat-free material.

The average weights of the live animals are also given. The dry, fat-free gland was taken as a basis for obtaining the exact proportion of active thymus tissue to the live weight of the animals. The calculated figures in the tabulation express the milligrams of dried fat-free gland tissue per kilo of body-weight, and demonstrate clearly that the proportion of thymus tissue is far larger in fetuses and in young growing animals than in fully mature animals.

The diet apparently has some influence on the size of the thymus gland, as it is considerably larger in the two species of herbivorous animals than in the omnivorous hog.

It was noticed that the fetal thymus glands were exceptionally rich in blood.

THYMUS GLANDS FROM	FRESH GLANDS									DESICCATED FAT-FREE MATERIAL				
	Average weight of animals		Total No. of glands		Average weight of fresh glands		Maximum weight of fresh glands		Minimum weight of fresh glands		Moisture			
	lbs.	gm.	gm.	gm.	per cent	per cent	per cent	per cent	mgm.	per cent	Ash per cent	Total N per cent	P <sub>2</sub> O <sub>5</sub> per cent	
Beef fetuses, 6-9 mos. old.....	40	268	55.1	180.0	17.0	79.2	0.3	20.5	19.7	598.0	4.12	10.15	14.41	6.40
Calves, 2-4 mos. old.	125	231	116.1	200.0	50.0	79.2	1.1	19.7	18.9	387.0	4.07	9.85	14.23	6.44
Full grown cattle .....	1120	120	188.0	345.0	70.0	69.6	14.2	16.2	15.6	57.7	3.51	8.95	14.29	5.93
Adult hogs .....	240	823	8.3	25.0	4.0	69.4	14.1	16.5	15.8	12.1	4.18	8.50	14.30	5.32
Lambs and sheep.....	80	754	13.3	54.0	11.0	78.8	4.6	16.6	16.1	59.0	3.18	9.05	14.43	5.78

The tabulated figures show very little difference in the moisture content between the fetal and calf thymus, and both lots are practically free from fat. The glands from full grown cattle and hogs are somewhat lower in moisture content, but show quite a high percentage of fat. The formation of adipose tissue in the interlobular or vascular connective tissue, which replaces the adenoid tissue containing the physiologically active constituents of the gland, is evidently very gradual and largely dependent on the age of the animal. This is borne out clearly in the case of sheep glands. Here we have all ages represented, and it is interesting to note that the moisture content is considerably higher than in adult cattle and hogs. The fat content is much lower than in the full grown animal, but is somewhat higher than in the fetal glands, and glands from young animals.

That the adipose tissue simply has replaced the adenoid tissue is shown by the almost identical yield of desiccated fat-free material obtained in all three cases where the full grown animals predominate.

On the desiccated, fat-free material the following determinations were made: moisture, ash, phosphoric acid, and total nitrogen. The moisture was determined on 5 gram samples of the powdered material in flat porcelain dishes by drying at 100°C. to constant weight. This required five hours. The ash was determined on 1 gram samples. The powder was weighed into tared porcelain crucibles of 15 cc. capacity and heated at a dull red heat in a muffle furnace to constant weight. This required approximately three hours.

The determinations of phosphorus were made according to the Neumann "wet" combustion method followed by titration of the yellow ammonium molybdate precipitate according to the details given in the volumetric method of the Association of Official Agricultural Chemists.

The total nitrogen was determined according to the official Kjeldahl-Gunning-Arnold method described in Bulletin 107, Bureau of Chemistry.

From the tabulated results it will be seen that the ash content of the thymus gland is high and consists principally of phosphates. The ash as well as the phosphorus contents are highest in glands from fetuses and very young animals, which, of course, strengthens

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the conclusion that the gland is most active and contains the highest percentage of active principle before puberty. There is but slight difference between the ash and phosphorus content of the fetal glands and glands from young calves. This indicates that the gland contains active principles and is therapeutically active during intra-uterine life.

If the figures found for total nitrogen are multiplied by the protein factor 6.25, it will be seen that the sum of the ash and protein is considerably above 100 per cent in all instances. This, of course, depends upon the fact that the thymus is very rich in nuclein bodies and relatively poor in ordinary proteids. It is interesting to note that the fetal glands also contain these nuclein bodies in quantities equal to those present in the glands from young animals. It appears, therefore, that the fetal thymus is in full activity at least three months before maturity.

Since the nuclein bodies are present in glands from full grown animals also, it is reasonable to assume that this gland does not cease its systemic activity throughout the growing and reproductive periods of the life of these animals.

### SUMMARY.

The fetuses and young growing animals contain very much more thymus tissue per unit of body-weight than fully mature animals.

The fetal thymus is exceptionally rich in blood and contains nuclein bodies and phosphates in amounts equal to those found in the glands from young growing animals, indicating that the gland is active therapeutically at least three months before maturity of the fetus.

Thymus glands from full grown animals also contain nuclein bodies and phosphates, indicating that the gland does not completely cease its systemic activity during the reproductive period of these animals.

Adult cattle and sheep (herbivora) contain more thymus tissue per unit of body-weight than adult hogs (omnivora).

## THE INFLUENCE OF A DIET OF MARINE ALGAE UPON THE IODINE CONTENT OF SHEEP'S THYROID.

BY ANDREW HUNTER AND SUTHERLAND SIMPSON.

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(Received for publication, December 21, 1914.)

It is a well known fact, emphasized afresh by each new series of analyses, that the thyroid gland, in respect to its iodine concentration, is subject to notable variations. This is true not merely for different species, but also for individuals within the species, and even for the same individual at different times. Among the factors responsible for these variations it is generally believed that the iodine content of the food plays the most important part. If this opinion be well founded, it would be expected that any group of animals subsisting upon a diet unusually rich in iodine should exhibit an unusual concentration of that element within its thyroids. We have recently, in the case of a group of sheep living under rather peculiar conditions, had an excellent opportunity of putting this expectation to the test; and we have found it, in that instance at least, to be fully realized. The observation seems to us of sufficient interest to be placed on record.

In many of the islands of the Orkney group, which lies to the north of Scotland, the native sheep run wild. They are of a small and hardy breed and during the winter months subsist, to a very large extent, on seaweed. Our material was obtained from one of these islands in the month of December. When the sheep were killed the thyroids were removed by a farmer, under the direction of the resident medical officer, and immediately transferred to 95 per cent alcohol, the gland of each animal (both lobes) being placed in a separate bottle. No record was kept of the body-weight, age, or sex of the animals, but all were adults. The bot-

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tles were sealed with paraffin to prevent evaporation, and the material was received by us, in good condition, about a month after it had been collected.

Each specimen received was removed from the alcohol in which it was preserved, thoroughly dried, and reduced to powder. Its iodine content was determined by the method described by one of us four years ago.<sup>1</sup> Whenever the amount of material permitted, the analysis was made in duplicate. The results are shown in Table I, where the glands are arranged in the order of their percentage iodine content.

TABLE I.

NO.	DRY WEIGHT OF GLAND	AMOUNT TAKEN FOR ANALYSIS	IODINE FOUND	IODINE FOUND	TOTAL IODINE OF GLAND
	gm.	gm.	mgm.	per cent	mgm.
1	0.45*	0.428	1.79	0.418	
2	0.54	0.527	2.63	0.499	2.70
3	1.25	{ 0.408 0.768	{ 2.23 4.31	{ 0.547 0.561	{ 0.554 6.94
4	0.81	{ 0.387 0.360	{ 2.37 2.19	{ 0.612 0.594	{ 0.603 4.89
5	1.15	{ 0.613 0.485	{ 4.16 3.24	{ 0.679 0.670	{ 0.674 7.74
6	1.15	{ 0.451 0.636	{ 3.19 4.52	{ 0.707 0.711	{ 0.709 8.15
7	0.66	0.654	5.15	0.788	5.20
8	0.97	0.546	4.72	0.864	8.37
9	1.37	{ 0.646 0.681	{ 5.96 6.40	{ 0.923 0.940	{ 0.931 12.77
10	0.94	{ 0.420 0.442	{ 4.35 4.70	{ 1.037 1.064	{ 1.050 9.87

\* One lobe only.

It appears from this table that in the glands under consideration the concentration of iodine ranges from 0.42 to 1.05 per cent of the dry substance, the average for the whole group of ten being as high as 0.709 per cent. The remarkable character of these figures is best revealed by comparison with the recent analyses of sheep's thyroid, which we have brought together in Table II.

<sup>1</sup> A. Hunter: this *Journal*, vii, p. 321, 1909-10.

TABLE II.

LOCALITY	NO. OF GLANDS	RANGE OF IODINE CONTENT	AVERAGE IODINE CONTENT	OBSERVER
		<i>per cent</i>	<i>per cent</i>	
Ohio.....	19	0.125-0.461	0.247	Marine and Lenhart. <sup>2</sup>
Western New York.....	10	0.048-0.383	0.168	Simpson and Hunter. <sup>3</sup>
Western New York.....	12	0.249-0.580	0.407	Simpson and Hunter. <sup>4</sup>
	Very large	0.049-0.260	0.158	Seidell and Fenger. <sup>5</sup>
Western New York.....	Very large	0.048-0.335	0.167	Seidell and Fenger. <sup>6</sup>
Newcastle-on-Tyne.....	Very large	0.280-0.510	0.375	Martin. <sup>7</sup>
Edinburgh....	Very large	0.222-0.279	0.251	Guyer. <sup>8</sup>

The last four sets of figures in Table II are derived from analyses, not of individual glands, but of composite powders obtained from groups of many hundreds. When, therefore, Martin reports an iodine content of 0.51 per cent, it is clear that his material must have included some glands that had an even higher percentage of the element. The figure 0.58 per cent observed by us in a sheep from Western New York could probably, therefore, be duplicated, if not surpassed, among sheep from Northern England. As it stands, however, it is the maximum value for sheep's thyroid hitherto recorded. It approaches closely the highest concentration of iodine thus far observed in any mammalian thyroid whatsoever; namely, 0.629 per cent for a canine thyroid analyzed by Marine and Lenhart.<sup>9</sup> Yet of the Orkney thyroids reported in this paper, no fewer than seven

<sup>2</sup> D. Marine and C. H. Lenhart: *Arch. Int. Med.*, iv, p. 440, 1909.

<sup>3</sup> S. Simpson and A. Hunter: *Quart. Jour. Exper. Physiol.*, iii, p. 121, 1910.

<sup>4</sup> Simpson and Hunter: *ibid.*, iv, p. 257, 1911.

<sup>5</sup> A. Seidell and F. Fenger: *Bull. Hyg. Lab., U. S. P. H. and M.-H. S.*, No. 96, p. 67, 1914.

<sup>6</sup> Seidell and Fenger: *this Journal*, xiii, p. 517, 1912-13.

<sup>7</sup> Martin: *Pharm. Jour.*, lxxxix, p. 144, 1912, and xci, p. 126, 1913. Quoted from Seidell and Fenger: *loc. cit.*, 1914.

<sup>8</sup> Guyer: *Pharm. Jour.*, xci, p. 123, 1913. Quoted from Seidell and Fenger: *loc. cit.*
<sup>9</sup> Marine and Lenhart: *loc. cit.*



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surpass the sheep, and six the mammalian record. Even the average for this group exceeds quite notably the previous maxima; while the series is closed by a gland containing the really astonishing quantity of over 1 per cent of iodine.

There is in the literature but one instance in which this last particular gland has been surpassed. It is supplied by Cameron<sup>10</sup> who found 1.16 per cent of iodine in a composite sample of thyroid powder from the elasmobranch fish, *Scyllium canicula*. That fish thyroids should frequently contain unusually large amounts of iodine is of particular interest in the present connection. Cameron connects his observation quite naturally with the constant presence of iodine in sea water; and in an extensive review of the distribution of iodine in plant and animal tissues,<sup>11</sup> he concludes that the variations in the iodine content of thyroid tissue are all referable to differences in diet. Without denying that other factors occasionally play a part,<sup>12</sup> we take our present results to be confirmatory of the general correctness of this view. While we have not identified nor analyzed the seaweeds consumed by our group of animals, there can be no doubt that they contained iodine.<sup>13</sup> That the sheep, like other animals, *can* store in its thyroid the iodine of its food is likewise beyond question; we have ourselves observed,<sup>14</sup> among other instances, one where the continued daily administration for six or seven weeks of small quantities of sodium iodide raised the iodine content of a sheep's thyroid from 0.53 to 1.15 per cent. There seems, therefore, to be no occasion to look beyond the food supply for an explanation of the analytical data we have presented.

<sup>10</sup> A. T. Cameron: *Biochem. Jour.*, vii, p. 466, 1913.

<sup>11</sup> Cameron: this *Journal*, xviii, p. 335, 1914.

<sup>12</sup> A discussion of factors accounting for seasonal variations will be found in Seidell and Fenger: *loc. cit.*, 1914.

<sup>13</sup> Our existing information upon the iodine content of different seaweeds is detailed by Cameron: this *Journal*, *loc. cit.*

<sup>14</sup> Simpson and Hunter: *Quart. Jour. Exper. Physiol.*, *loc. cit.*

# **A NOTE ON THE DISTRIBUTION OF MERCURY IN THE BODY IN A CASE OF ACUTE BICHLORIDE OF MERCURY POISONING.**

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(Received for publication, December 21, 1914.)

The writer recently assisted in an autopsy on the body of a young girl who had, ten days previously, taken about 15 grams of bichloride of mercury for suicidal purposes. The autopsy was held about five hours after death, and it was thought that it would be of value to estimate the amount of mercury contained

ORGAN	MERCURY IN MG.M. PER 100 GM. OF ORGAN	MERCURY IN MG.M. IN WHOLE ORGAN
Kidney.....	2.80	5.80
Spleen.....	1.20	1.70
Liver.....	3.62	39.82
Brain.....	0.14	1.68
Stomach.....	0.50	1.50
Small intestine.....	1.60	4.80
Large intestine.....	1.82	7.28
Heart.....	1.92	5.81
Lungs.....	Trace	Trace
Blood.....	1.20	52.0*
Muscle.....	0.06	25.8†
Bile.....	Trace	Trace
Stomach contents.....		12.20
Intestinal contents.....		8.60
Rectal contents.....		8.40
Total.....		175.39

\* Calculated on the basis of the blood constituting  $\frac{1}{3}$  of the body-weight.

† Calculated on the basis of the muscle constituting  $\frac{1}{2}$  of the body-weight.

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in various organs of the body.<sup>1</sup> The mercury content of the organs was estimated by Ludwig's method.<sup>2</sup>

The above tabulated data give the amounts of mercury found in the various organs and fluids of the body. They show that the liver contains the largest amount of mercury per 100 grams of tissue, but that the blood contains the largest quantity based on the whole amount present in the body.

The mercury found in the contents of the stomach, intestines, and rectum may, of course, represent mercury absorbed and reëxcreted into those channels.

<sup>1</sup> A thorough review of the literature on the distribution of mercury in the body may be found in Witthaus and Becker: *Medical Jurisprudence, Forensic Medicine and Toxicology*, iv, p. 721, 1911.

<sup>2</sup> E. Ludwig: *Ztschr. f. anal. Chem.*, xvii, p. 395, 1878 (cited by C. Neubauer); xx, p. 475, 1881 (cited by F. Hofmeister); *Wien. med. Presse*, xxxiii, p. 1891, 1892; E. Ludwig and E. Zillner: *Wien. klin. Wchnschr.*, ii, p. 858, 1889; iii, p. 534, 1890.

## ON THE ESTIMATION OF BENZOIC ACID IN URINE.

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(Received for publication, December 28, 1914.)

For the quantitative estimation of free benzoic acid in the urine of rabbits we have found that none of the methods described in the literature are entirely satisfactory in the points of convenience and rapidity. Recently, Steenbock<sup>1</sup> has reviewed the methods used for the determination of benzoic and hippuric acids, recommending Dakin's method for isolating benzoic acid, with further purification by subliming according to his own modification. Soon afterwards, Folin and Flanders<sup>2</sup> published their method for the determination of hippuric acid, which is, because of its convenience, superior to any previously described. According to this method, the hippuric acid is hydrolyzed into benzoic acid and glycocholl by first evaporating the urine with alkali on the water bath, and then boiling for several hours with nitric acid and copper nitrate. The resulting benzoic acid is extracted with chloroform and titrated with  $\frac{N}{10}$  sodium alcoholate.

The extraction of free benzoic acid from fresh urine with chloroform would be feasible, if it were not for the troublesome emulsions formed. Working with the urine of rabbits which had received large amounts of benzoic acid, we could get no satisfactory results, chiefly because of the great extent to which the chloroform emulsified. Heating or any other treatment of the urine with the purpose of removing the emulsifying substances, resulted in splitting of hippuric acid or the loss of benzoic acid.

Consequently we have sought a solvent of benzoic acid which would form the least emulsion when shaken with fresh urine.

<sup>1</sup> H. Steenbock: *this Journal*, xi, p. 201, 1912.

<sup>2</sup> O. Folin and F. F. Flanders: *this Journal*, xi, p. 257, 1912.

Out of many solvents tried, toluene appeared to be the best. While it is an excellent solvent for benzoic acid, it practically does not dissolve hippuric acid, and is superior to chloroform in many ways.<sup>3</sup> As a rule, toluene does not produce emulsions when shaken with fresh urines, but small emulsions may form when working with abnormal urines; for example, those contaminated with feces. In such cases it was found that the emulsion could be removed by shaking with a small amount of absolute alcohol.

Our method is a modification of that proposed by Folin and Flanders<sup>4</sup> for ketchup. The procedure is as follows: 100 cc. of fresh urine are pipetted into a 500 cc. short stemmed<sup>5</sup> separatory funnel, and acidified with 1 cc. of concentrated nitric acid. Enough ammonium sulphate (50 to 60 gm.) to saturate the urine is added, and the benzoic acid is extracted with four portions of pure toluene<sup>6</sup> of 50, 40, 30, and 30 cc. each, respectively. The combined toluene extracts are then washed twice, using 100 cc. each time, with saturated sodium chloride solution, containing in each liter 0.5 cc. of concentrated hydrochloric acid. The titration is made with  $\frac{N}{10}$  or  $\frac{N}{20}$  sodium alcoholate,<sup>7</sup> using phenolphthalein as an indicator. The end-point is a definite pink, lasting two or three minutes.

To secure the best results, the following points should be observed. The contents of the funnel are shaken with a rotary motion, from 15 to 20 times with each portion of toluene. If the shaking is not too vigorous, no emulsion should occur. Should there be a slight emulsion, however, it is disregarded for the present, and obviated in a later stage of the analysis by addition of absolute alcohol. After the first extraction the urine is drawn off into a second funnel, leaving behind the toluene and any

<sup>3</sup> One of the advantages of toluene is that it does away with the discomforts so often experienced while working with chloroform.

<sup>4</sup> Folin and Flanders: *Jour. Am. Chem. Soc.*, xxxiii, p. 1622, 1911.

<sup>5</sup> The stem of an ordinary separatory funnel is cut off about an inch from the stop-cock.

<sup>6</sup> The toluene used for extractions may be distilled and washed twice with distilled water, when it is again ready for use.

<sup>7</sup> Sodium alcoholate is prepared by dissolving 2.3 gm. of clean metallic sodium in 1 or 2 liters, respectively, of absolute alcohol. This solution is standardized against pure benzoic acid dissolved in toluene.

emulsion that may have formed; the urine is then shaken with the second portion of the toluene.

In order to avoid the use of a third funnel at this point, the urine is drawn off into an Erlenmeyer flask, and the toluene run into the funnel containing the first extract. The urine is then transferred back again into the second funnel, washing the flask with the required amount of toluene for the third extraction. The fourth extraction is carried out in a similar manner.

Before washing the combined extracts, contained in the first funnel, with sodium chloride solution, any urine that may have collected is drawn off. If there is emulsion present, 1 cc. of absolute ethyl alcohol is added, and the funnel, held in an upright position, is rotated vigorously. Drops of urine and solid matter settle to the bottom and are drawn off. After washing twice with sodium chloride solution, the funnel is again rotated vigorously for the reason just mentioned. The stem of the funnel is now washed with water, dried with a piece of filter paper, and the toluene drawn off into a dry flask and titrated.

In order to show that toluene extracts benzoic acid from watery solution quantitatively, 0.050 gm. and 0.100 gm. of pure benzoic acid were dissolved by means of sodium hydrate in 100 cc. of water. The titrations obtained with sodium alcoholate (1 cc. = 0.0161 gm. of benzoic acid) were 3.00 cc. and 3.05 cc. in one case, and 6.05 cc. and 6.10 cc. in the other. Hence the amount of benzoic acid recovered was 0.049 gm. and 0.098 gm., respectively.

Four analyses of human urine, made according to the method described, showed the absence of benzoic acid, thus confirming the findings of others, and also indicating that toluene does not extract any substances from the urine, except benzoic acid, which would neutralize sodium alcoholate.

In the accompanying table the results of a number of experiments on rabbit urines are recorded. In Column 1 the amount of benzoic acid found in the urine (preformed) is given. Column 2 records the amount of benzoic acid added to the different urines. Column 3 shows the amount of total free benzoic acid found, and Column 4 gives the amount of added benzoic acid which was recovered. In Column 5 the recovered benzoic acid is calculated on a percentage basis. From the figures we see that the amount of benzoic acid recovered is practically quantitative. To

some of these urines 0.100 gm. of hippuric acid was added, in addition to the benzoic acid, without affecting the result, showing that none of the hippuric acid was extracted by toluene.

TABLE I.

URINE	(1)	(2)	(3)	(4)	(5)	(6)
	BENZOIC ACID FOUND IN 100 CC. OF URINE BEFORE ADDITION	BENZOIC ACID ADDED TO 100 CC. OF URINE	BENZOIC ACID FOUND IN 100 CC. OF URINE AFTER ADDITION	BENZOIC ACID RECOV- ERED	BENZOIC ACID RECOV- ERED	HIPPURIC ACID ADDED
	gm.	gm.	gm.	gm.	per cent	gm.
A.....	0.0021	<b>0.0423</b>	0.0443	<b>0.0422</b>	99.8	
	0.0018	<b>0.0423</b>	0.0444	<b>0.0426</b>	100.5	
	0.0019	<b>0.0423</b>	0.0449	<b>0.0430</b>	101.5	
B.....	0.0065	<b>0.0846</b>	0.0887	<b>0.0822</b>	97.2	0.100
	0.0072	<b>0.0846</b>	0.0894	<b>0.0822</b>	97.2	0.100
C*.....	0.0920	<b>0.100</b>	0.1900	<b>0.0980</b>	98.0	0.100
	0.0912	<b>0.100</b>	0.1885	<b>0.0973</b>	97.3	0.100
D*.....	0.0330	<b>0.1046</b>	0.1368	<b>0.1038</b>	99.3	
	0.0338	<b>0.1060</b>	0.1377	<b>0.1039</b>	99.0	

\* This rabbit had received large amounts of benzoic acid *per os*.

## STUDIES ON THE THEORY OF DIABETES.

### IV. THE PARALLELISM BETWEEN THE EFFECTS OF THE PANCREAS AND THOSE OF METALLIC HYDROXIDES ON SUGARS.

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(Received for publication, December 28, 1914.)

When in any case of pancreas or phlorhizin diabetes any sugar, such as galactose, levulose, mannose, etc., is given under conditions so selected that the sugar almost wholly escapes oxidation, such sugar may still be fully transformed into *d*-glucose. This phenomenon serves to demarcate two distinct types of sugar reaction in the body: the *destructive* and the *transformative*. *It is only the former of these which disappears in diabetes.* In a study of the fundamental chemical nature of diabetes it is important to discriminate between these and to ascertain, if possible, the chemical nature of each and the laws which govern them. Some light is thrown on the subject by a consideration of certain purely chemical properties of sugars.

Sugars in alkaline solutions *in vitro* manifest the same two types of reaction, and in this field the phenomena have been extensively investigated and illuminated. It has long been known that when a solution of any monosaccharose, such as *d*-glucose, is alkalinized, the rotatory power of the solution falls and finally disappears, the solution then containing a mixture of hexoses in a state of dynamic chemical equilibrium as shown by Lobry de Bruyn and van Ekenstein.<sup>1</sup> Knowledge of the probable number and character of the substances participating in such an equilibrium has been extended by Nef.<sup>2</sup> If the conditions of the experiment are suitable (very dilute alkali), there need be no browning of the solution nor any

<sup>1</sup> Lobry de Bruyn and van Ekenstein: *Rec. trav. chim. de Pays Bas*, xiv, pp. 158, 203; xv, p. 92; xvi, p. 257; xix, pp. 1, 10.

<sup>2</sup> J. U. Nef: *Ann. d. Chem.*, ccclvii, p. 214, 1907.



detectable loss of total reducing power whatsoever. *These reciprocal transformations without loss of reducing power will be referred to as reactions of type I.* With stronger concentrations of alkali, loss of reducing power occurs. In the absence of oxygen or oxidizing agents this loss is associated with the formation of lactic acid, and the saccharinic acids, and browning (tars). In the presence of sufficient oxygen (air,  $\text{H}_2\text{O}_2$ , metallic oxides, etc.), oxidation products occur instead of the former. *These destructive reactions will be referred to as type II.* According to Michaelis and Rona,<sup>3</sup> loss of reducing power was first seen in 1 to 5 per cent glucose solutions (with air excluded) when the OH ion concentration corresponded to  $(\text{H} \cdot) = 10^{-11}$  or  $10^{-12}$ ; whereas, in a certain instance, loss of rotatory power was seen with  $(\text{H} \cdot) = 7 \times 10^{-11}$ . In other words, reactions of type I may prevail with a certain low OH ion concentration, those of type II appearing only with higher OH ion concentrations. It is of interest to inquire *how* a metallic hydroxide acting as a catalytic agent produces these effects.

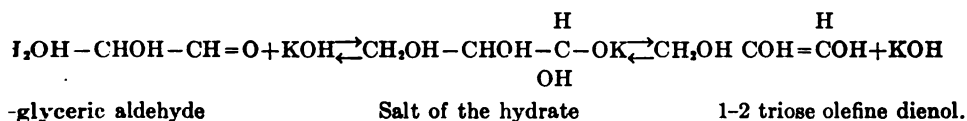
Sugars are weak acids. They form salts with metals and influence the electro conductivity of weak electrolytes as acids should. The dissociation constants of several sugars have been measured by electro chemical methods. Cohen<sup>4</sup> found for the dissociation constant of *d*-glucose as an acid  $6 \times 10^{-13}$ . Michaelis and Rona found  $5 \times 10^{-13}$ . In 1909 A. P. Mathews<sup>5</sup> noted that the speed of oxidation of sugars in the presence of alkali was proportional up to a certain point with the concentration of the alkali, and proposed that metallic hydroxides acting after the manner of strong bases on weak acids in general formed salts with high electrolytic dissociation constants leading to an increased concentration of sugar anions. These by virtue of a partially unbalanced charge are unstable and readily rearrange, oxidize, etc. Michaelis and Rona demonstrated that the rate of decline of rotatory power in glucose solutions was proportional to the OH ion concentration. They proposed that, in accordance with mass laws, the rising OH ion concentration increases the play between sugar molecules and ions as an incident to which new molecules appear; such, for

<sup>3</sup> L. Michaelis and P. Rona: *Biochem. Ztschr.*, xlix, p. 232, 1913; xlvii, p. 447, 1912.

<sup>4</sup> E. Cohen: *Ztschr. f. physikal. Chem.*, xxxvii, p. 69, 1901.

<sup>5</sup> A. P. Mathews: *this Journal*, vi, p. 3, 1909.

example, as enols of the type proposed by Wohl and Neuberg. Nef ascribes reactions of type I to dissociations occurring primarily at the aldehyde or ketone groups, the double bond between carbon and oxygen opening after the manner of simpler aldehydes and ketones to form hydrates with water. These hydrates acting like alcohols form salts which readily dissociate with the formation of an olefine dienol. The mechanism in the case of glyceric aldehyde would be as follows:



The 1-2 triose olefine dienol is derivable from either *d*- or *l*-glyceric-aldehyde or from dihydroxy acetone, and once formed from any of these the hydrates of all of the three trioses may be reformed from the enol by the simple addition of H and OH in the different ways permitted by the laws of space chemistry. The metallic hydroxides acting as electrolytes lead to preliminary salt formation, the salts then dissociating like the original substances, but to a far greater degree.

For present purposes it is unnecessary to enter into a detailed discussion of the relative advantages of these views. It may be said that they agree in the essential that metallic hydroxides, acting as electrolytes, on electrolytically dissociated sugars, initiate the production of an increased concentration in the solution of certain dissociated fragments of sugar; that these fragments are highly susceptible to chemical change and are in a state of dynamic chemical equilibrium with the undissociated sugar molecules. It is clear too that secondary intramolecular changes of a character not identifiable as electrolytic in the usual sense, follow the initial electrolytic reaction in any case. Nef's methylene chemistry begins at this point.

The fact that a parallelism exists between the behavior of sugars in the body and in an alkaline solution in the test-tube does not of itself prove that the reactions in each case depend on a fundamentally identical mechanism. Nevertheless there is a hint given which it would be folly to ignore, and if for the explanation of the laboratory sugar reactions conceptions involving dissociation and equilib-

rium have been found necessary, they can hardly be left out of consideration in the interpretation of the phenomena of carbohydrate metabolism. Moreover, the application of such chemical conceptions at once simplifies and broadens our view of many metabolic processes, and we may be permitted to sketch briefly a possible application to the problem of diabetes.

In diabetes mellitus there is an excess of sugar in the blood and still reactions of type II fail, while those of type I persist, and by excessive feedings of *d*-glucose the conditions are relatively little altered, provided the case is one of genuinely total diabetes. This is quite parallel to what occurs in a sugar solution in which the alkali is sufficiently dilute. In such cases of diabetes there is a lack of the "something derived from the pancreas," and it is a short step to propose that this something does for sugar what *in vitro* is accomplished by alkali sufficiently concentrated.<sup>6</sup> According to this interpretation we have in diabetic blood an hyperglycemia only of molecular or undissociated inert sugar, but an hypoglycemia as regards active dissociated sugar of the kind necessary for oxidative or type II reactions.

In phlorhizin diabetes there is an absolute hypoglycemia as regards sugar of all forms. Here also reactions of type II disappear. But in this case the feeding of glucose in high concentration causes the reappearance of oxidative reactions, so that here the equivalent of alkali derived from the pancreas cannot be regarded as lacking. Is it not possible that, as a part of the general hypoglycemia, the concentration of dissociation residues of the kind necessary for type II reactions is also low, and that diabetes mellitus and phlorhizin diabetes have in common a low blood and tissue concentration of certain forms of dissociated sugar? This would account for the identity of the metabolic sequences which follow the development of either kind of diabetes, provided that the under-

<sup>6</sup> So far the study of body fluids and tissues by physico-chemical methods has failed to reveal such concentrations of OH ions as would be necessary accompaniments of reactions of either type I or II in a simple aqueous solution outside the body. The above statement in the text is not intended to imply that sugar dissociation is necessarily accomplished in the body by alkaline hydroxides or OH ions, but rather by those substances or that substance which under physico-chemical conditions found in the cell exert on sugars an effect equivalent to that of metallic hydroxides in a watery solution.

lying hypoglycemia of phlorhizin diabetes is not obliterated by a too rapid entry of sugar into the blood from without, or by a blocking of the excretion of sugar through the kidneys.<sup>7</sup>

In this connection it is always borne in mind by the writer that the pancreas is a member of the group of so called endocrinous glands, and that if its internal secretion acts as a dissociating agent for a certain class of metabolites it would be reasonable to expect that some other internal secretions behave in a similar way with other classes of metabolites. Thus the thyroid may be concerned with the dissociation of amino-acids or polypeptides derived from the breakdown of protein. There is evidence bearing on this subject which cannot be given here. The possibility is clearly presented that between certain breakdown products of fats, protein, and carbohydrates, common dissociation residues or systems of residues may exist which throw these classes of substances into an actual state of organic chemical equilibrium with one another, —a conception which bears directly on the nature of the correlation of the ductless glands and in a most tangible manner, which we hope to detail in later communications. This idea also bears on the question of how carbohydrates or fats suppress protein catabolism, etc.

<sup>7</sup> In using the terms "blood" and "kidneys" the writer is not unconscious of the fact that cells in general must be able to accomplish within themselves that which blood and kidneys do for the body as a whole, and that the excretion of sugar from a tissue may be blocked without injury to the kidneys. Also, the blood is merely one tissue.



## CONDITION OF CASEIN AND SALTS IN MILK. X

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### INTRODUCTION.

The chemistry of milk has been studied by many investigators. Numerous facts have been accumulated relative to the amounts and properties of the more prominent constituents of milk, including various conditions affecting the composition; but much less attention has been given to thorough study of individual constituents, owing largely to the difficulties involved in making such investigations.

From the beginning of its existence, this Station has given much attention to study of different phases of the composition of milk. In connection with the study of the relation of the constituents of milk to cheese-making, to fermented beverages made from milk, and to the uses of milk in human nutrition, numerous chemical questions have constantly arisen and continue to come up, to which satisfactory answers can not be given, owing to our lack of knowledge of the chemistry of some of the milk constituents. Until our knowledge in this field becomes more complete, we can not understand fully, for example, the fundamental chemical facts involved in the process of cheese-making and cheese-ripening, the chemical changes taking place in its constituents when milk sours or when it is made into fermented beverages, such as kumyss, imitation buttermilk, matzoon, zoolak, bulgarzoon, etc.

We have in hand investigations relating to several of the fundamental questions referred to. In the present bulletin we shall present the results of our work bearing on the following points:

(1) Properties and composition of milk serum or constituents in solution.

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- (2) Properties and composition of portion of constituents not in solution.
- (3) Acidity of milk and milk serum.
- (4) The salts of milk.

### METHOD OF PREPARING MILK SERUM.

Before taking up the detailed results relative to these lines of investigation, we will give a description of the method used in preparing milk serum from milk.

That portion of the milk consisting of water and the compounds in solution is known as the milk serum. In studying the individual constituents of milk, it is necessary to separate the serum. Various methods have been used to separate milk serum from the other constituents of milk, but the one best adapted for investigational purposes depends upon the fact that when milk is brought into contact with a porous earthenware filter, the water passes through, carrying with it the compounds in true solution, while the compounds insoluble in water, or in suspension, remain on the surface of the filter. In one form or another, this fact has been utilized in studying milk by Lehman, Duclaux, Eugling, Söldner, and others. The form of earthenware filter used by us is much superior to any employed by these investigators. We have made use of the special form of apparatus designed by Briggs<sup>1</sup> for the purpose of obtaining water extracts from soils. Briefly stated, the process consists in putting the milk to be examined into a tubular chamber surrounding a Pasteur-Chamberland filtering tube; pressure, amounting to 40 to 45 pounds per square inch, is applied by means of a pump which forces air into the chamber containing the milk and causes the soluble portion of the milk to pass through the walls of the filter from the outside to the inside of the filter tube, from which it runs out and is caught in a flask standing underneath. The insoluble residue accumulates on the outside surface of the filter tube, from which it can easily be removed by light scraping.

<sup>1</sup> L. J. Briggs and M. H. Lapham: *U. S. Department of Agriculture, Bureau of Soils, Bull. 19*, p. 31, 1902; O. Schreiner and G. H. Failyer: *idem, Bull. 31*, pp. 12-16, 1906.

It has been found by Rupp<sup>2</sup> that the filter appears to have the power of adsorbing some of the constituents of the serum until a volume of 50 to 75 cc. has passed through, after which the filtered serum is constant in composition. In our work, therefore, the first portion of serum filtered is not used.

Before being placed in the apparatus for filtration, the milk is treated with some antiseptic to prevent souring during the process of filtration.

The composition of the solid portion of milk removed by the filtering tube is ascertained by difference; from the figures obtained by an analysis of the original milk we subtract the results of analysis given by the serum.

*Properties and composition of milk serum.*

Serum prepared from fresh milk by the method described above has a characteristic appearance, being of a yellow color with a faint greenish tinge and slight opalescence.

The serum from fresh milk gives a slight acid reaction to phenolphthalein and a strongly alkaline reaction to methyl orange. We will later give the result of a special study made of the cause of acidity in milk serum.

In the table below we give the results of the examination of two samples of fresh milk, the serum of which was prepared in the manner already described. These samples of milk were treated with chloroform at the rate of 50 cc. per 1000 cc. of milk, and the fat was removed by means of a centrifugal machine; the removal of fat is necessary since it clogs the pores of the filter. The fat-free milk was then filtered through Pasteur-Chamberland filtering tubes. Analyses were made of the milk and of the serum. We did not determine those constituents present in milk only in traces, such as iron, sulphuric acid, etc.

A study of the data contained in Table I enables us to show the general relation of the constituents of milk to the constituents of milk serum. The following form of statement furnishes a clear summary of the facts:

<sup>2</sup> P. Rupp: *U. S. Department of Agriculture, Bureau of Animal Industry, Bull. 166*, p. 9, 1913.



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1. Milk constituents  
in true solution  
in milk serum.

- (a) Sugar
- (b) Citric acid
- (c) Potassium
- (d) Sodium
- (e) Chlorine

2. Milk constituents  
partly in solu-  
tion and partly  
in suspension or  
colloidal solu-  
tion.

- (a) Albumin
- (b) Inorganic phosphate
- (c) Calcium
- (d) Magnesium

3. Milk constituents  
entirely in sus-  
pension or col-  
loidal solution.

- (a) Fat.
- (b) Casein.

TABLE I.  
*Constituents of milk serum.*

CONSTITUENTS	SAMPLE 1			SAMPLE 2		
	Original milk 100 cc.	Milk serum 100 cc.	Percentage of milk con- stituents in serum	Original milk 100 cc.	Milk serum 100 cc.	Percentage of milk con- stituents in serum
	gm.	gm.		gm.	gm.	
Sugar.....				5.75	5.75	100.00
Casein.....	3.35	0.00	0.00	3.07	0.00	0.00
Albumin.....	0.525	0.369	70.29	0.506	0.188	37.15
Nitrogen in other compounds.....				0.049	0.049	100.00
Citric acid.....				0.237	0.237	100.00
Phosphorus (organic and inorganic)	0.125	0.067	53.60			
Phosphorus (inorganic).....	0.096	0.067	70.00	0.087	0.056	64.40
Calcium.....	0.128	0.045	35.16	0.144	0.048	33.33
Magnesium.....	0.012	0.009	75.00	0.013	0.007	53.85
Potassium.....	0.354*	0.352*	99.44	0.120	0.124	100.00
Sodium.....				0.055	0.057	100.00
Chlorine.....	0.081	0.082	100.00	0.076	0.081	100.00
Ash.....				0.725	0.400	55.17

\* As chlorides.

The behavior of milk albumin attracts special attention on account of marked lack of regularity in the results obtained. We commonly think of milk albumin as readily and completely soluble in water, and the question is therefore raised as to why a considerable portion of it does not pass through the Pasteur-Chamberland filter. In view of all the facts available, the most probable explanation that has so far suggested itself is that in fresh milk a part of the albumin is held by the adsorbing power of casein. This suggestion is supported by results obtained in the

following experiments: Serum was prepared from chloroformed fresh milk treated in different ways. In Experiment 1 serum direct from the fresh milk was compared with serum obtained from whey which had been obtained from another portion of the same milk by treatment with rennet extract. In Experiment 2 serum direct from fresh milk was compared with (a) serum obtained from another portion of the same milk after souring and (b) serum obtained from another portion of the same milk to which some formaldehyde solution had been added. Albumin was determined in each case by boiling after addition of acetic acid, following the details given in the provisional method of the Association of Official Agricultural Chemists. The results of the experiments are given below.

## EXPERIMENT 1.

	ALBUMIN PER 100 CC. gm.	ALBUMIN OF MILK RECOVERED IN SERUM per cent
Fresh milk.....	0.312	
Serum from fresh milk.....	0.143	45.83
Serum from whey.....	0.187	59.94

## EXPERIMENT 2.

Fresh milk.....	0.266	
Serum from fresh milk.....	0.148	55.64
Serum from sour milk.....	0.253	95.11
Serum from milk plus formaldehyde.....	0.245	92.21

In Experiment 1 it is seen that when casein is precipitated by rennet solution the curd (the precipitated casein or paracasein) carries down part of the albumin with it; the amount thus carried down is approximately equal in this case to that retained along with the casein on the external surface of the Pasteur-Chamberland filtering tube, when whole milk is filtered through such a filter.

In Experiment 2 we see that when the casein is precipitated with acid, as in the case of natural souring, the adsorbing action of the casein is practically prevented and little or no albumin is carried down with it. In the case of the addition of formaldehyde to milk, the adsorbing power of casein is greatly diminished, probably due to the chemical reaction between casein and formaldehyde.

*Properties and composition of portion of milk in suspension or colloidal solution.*

Some of the constituents of milk are suspended in the form of solid particles in such an extremely fine state of division that they pass through the pores of filter paper, and they do not settle as a sediment on standing, but remain permanently afloat, and they cannot be seen except by ultramicroscopic methods. When substances are in such a condition, they are said to form a colloidal solution. In passing milk through the Pasteur-Chamberland filtering tube, the constituents in suspension as solid particles, that is, in colloidal solution, are retained in a solid mass on the outside of the tube and can therefore be readily obtained for study.

(1) *Appearance.* When prepared by the method of filtration previously described, the insoluble portion of milk collecting on the outside of the filtering tube is grayish to greenish white in color, of a glistening, slime-like appearance, and of gelatinous consistency. When dried, without purification by treatment with alcohol, etc., it resembles in appearance dried white of egg.

(2) *Behavior with water.* The deposit of insoluble milk constituents on the outside of the filtering tube, when removed and shaken vigorously in a flask with distilled water, goes into suspension, and the mixture has the opaque, white appearance of the original milk. The deposit is, of course, more or less mixed with adhering soluble constituents, but can be readily purified by shaking with distilled water and filtering several times. The purified material goes readily into suspension on shaking with water and, if treated with a preservative, will remain indefinitely without change other than the separation of fat globules. It has been held by some that the citrates of milk perform the function of holding the insoluble phosphates in suspension, but this is not supported by the behavior of the insoluble portion shown in our experiments.

(3) *Reaction.* A suspension of the insoluble constituents of milk, prepared in the manner described above, is neutral to phenolphthalein. We purified the deposit made from 1000 cc. of milk, made a suspension of it in water, and, after the addition of 10 cc. of neutral solution of potassium oxalate, it was found to require only 0.5 cc. of  $\frac{N}{10}$  solution of sodium hydroxide to make it

neutral to phenolphthalein. We interpret this to mean that there are no tri-basic (alkaline) phosphates in milk or in the serum; because the serum, since it is acid, can contain none, and the insoluble portion, being neutral, can therefore contain none.

(4) *Relation of inorganic constituents to casein in milk.* Without going into a detailed discussion of the history of the different views held by investigators, it is sufficient for our purpose to state that three general views have been put forward in regard to the relation of inorganic constituents to casein in milk: (1) That milk casein is combined with calcium (about 1.07 per cent) to form a salt, calcium caseinate (which is neutral to litmus and acid to phenolphthalein); (2) that casein is chemically combined directly with calcium phosphate; (3) that casein is a double compound consisting of calcium caseinate combined with calcium phosphate.

We have attempted to learn what is the true condition of casein in milk in relation to inorganic constituents, whether it is in combination with calcium alone or with some other inorganic base in addition, and also whether milk casein is an acid salt or a neutral salt, and, further, whether the insoluble phosphates are in combination with casein or not.

In studying the problem, we will first give the results of work done with sixteen samples of fresh milk from thirteen individual cows. The different determinations made are as follows: (a) casein, (b) total phosphorus, (c) soluble phosphorus, (d) insoluble phosphorus (b minus c), (e) insoluble organic phosphorus (casein multiplied by 0.0071), (f) insoluble inorganic phosphorus (d minus e), (g) total calcium, (h) soluble calcium, (i) insoluble calcium (g minus h), (j) total magnesium, (k) soluble magnesium, (l) insoluble magnesium (j minus k). The determinations of casein, total phosphorus, total calcium, and total magnesium were made with the normal or whole milk, while those of soluble phosphorus, soluble calcium, and soluble magnesium were made with the serum obtained by filtering through Pasteur-Chamberland filtering tubes in the manner already described. The amount of organic phosphorus was found by multiplying the percentage of casein by 0.0071.<sup>3</sup> For convenience of reference, the analytical data are arranged in Tables II and III.

<sup>3</sup> A. W. Bosworth and L. L. Van Slyke: this *Journal*, xix, p. 67, 1914.

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The data in Table II afford a basis for ascertaining the quantitative relation between casein and the phosphates. If casein is chemically combined with phosphates in milk, there should be a fairly definite and uniform relation between these constituents in the insoluble portion of milk, or, stated in another way, the organic phosphorus of casein should show a somewhat uniform

TABLE II.  
*Amounts of proteins, casein, and phosphorus in milk.*

COW NO.	STAGE OF LACTATION	TOTAL PROTEIN	CASEIN	PHOSPHORUS					RATIO OF ORGANIC TO INSOLUBLE INORGANIC PHOSPHORUS
				Total Soluble		Insoluble			Organic P: inorganic P
				Total		Total	Organic (in casein)	Inorganic (phosphates)	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	
1.....	3 dys.	4.35	3.48	0.1272	0.0818	0.0454	0.0247	0.0207	1: 0.83
2.....	1 mo.	3.31	2.73	0.1150	0.0395	0.0555	0.0194	0.0361	1: 1.86
3.....	1 mo.	3.53	2.78	0.1210	0.0494	0.0516	0.0197	0.0319	1: 1.62
3.....	11 mo.	4.91	4.09	0.1111	0.0536	0.0575	0.0290	0.0285	1: 0.98
4.....	3 mo.	3.93	3.09	0.1278	0.0563	0.0715	0.0219	0.0496	1: 2.26
5.....	3 mo.	3.45	2.88	0.0943	0.0475	0.0468	0.0204	0.0264	1: 1.29
5.....	7 mo.	3.45	2.70	0.0870	0.0334	0.0536	0.0192	0.0344	1: 1.79
6.....	5 mo.	4.05	2.92	0.1008	0.0356	0.0652	0.0207	0.0445	1: 2.15
7.....	6 mo.	4.07	3.40	0.1063	0.0548	0.0515	0.0241	0.0274	1: 1.14
7.....	10 mo.	4.80	3.56	0.1010	0.0340	0.0670	0.0253	0.0417	1: 1.65
8.....	7 mo.	4.39	3.58	0.1157	0.0550	0.0607	0.0254	0.0353	1: 1.39
9.....	8 mo.	4.33	3.47	0.1036	0.0364	0.0672	0.0246	0.0426	1: 1.73
10.....	9 mo.	3.65	3.10	0.1097	0.0610	0.0487	0.0220	0.0267	1: 1.22
11.....	10 mo.	4.17	3.36	0.1090	0.0434	0.0656	0.0239	0.0417	1: 1.74
12.....	11 mo.	4.35	3.14	0.1060	0.0286	0.0774	0.0223	0.0551	1: 2.47
13.....	12 mo.	5.71	4.97	0.1310	0.0442	0.0868	0.0353	0.0515	1: 1.46

ratio to the insoluble inorganic or phosphate phosphorus. In column 10 of Table II we give the results of calculations based on our data, which show the amount of insoluble inorganic phosphorus for one part of organic (casein) phosphorus. It is seen that the ratio varies between the wide limits of 1:0.83 and 1:2.47. Even in the case of milk from the same animal at different stages of lactation, the proportional amounts of inorganic phosphorus vary widely, as from 0.98 to 1.62 with Cow 3, from

1.29 to 1.79 with Cow 5, and from 1.14 to 1.65 with Cow 7. The only conclusion furnished by these results is that there is no evidence of chemical combination between the casein and the phosphates of milk. Additional evidence in confirmation of the foregoing statement will be furnished later in connection with the discussion of another phase of the subject.

Another interesting point connected with insoluble phosphates and casein in milk is as to the exact compound of calcium phosphate and of calcium caseinate existing in the milk. Söldner's inferential statement that milk casein is neutral calcium caseinate (containing about 1.07 per cent of calcium), has been generally accepted, not so much because of positive proof but because of absence of any proof to the contrary. Regarding the form of the compound in which phosphates exist in milk, all three forms (mono-, di-, and tri-basic phosphates) have been thought to be present. The insoluble phosphates have been regarded as a mixture of di- and tri-calcium phosphate. Bearing on this question, we present data embodied in Tables III and IV.

The data in Table IV are derived by calculation from the figures given in Tables II and III, for the purpose of reducing them to a uniform basis that permits us to make comparison more easily.

In our previous work we have shown that one gram of uncombined casein combines with  $9 \times 10^{-4}$  gram equivalents of calcium to form a salt that is neutral to phenolphthalein.<sup>4</sup> In column 2 of Table IV we use this fact in calculating the acid equivalents of the casein as found in each sample. In column 3 we calculate the acid equivalents of the insoluble inorganic phosphorus in each sample of milk (regarding phosphoric acid as a di-valent acid and  $\text{CaHPO}_4$  neutral to phenolphthalein). In column 4 we give the sums obtained by adding the figures in columns 2 and 3 in the case of each sample of milk. In columns 5 and 6 we give the combining equivalents of calcium and magnesium, and in column 7 their sums for each sample of milk. If now we compare in the case of each milk the figures contained in column 4 with those contained in column 7, we notice that they are in close agreement, the differences being shown in column 8. This agreement means that the quantitative

<sup>4</sup> A. W. Bosworth and L. L. Van Slyke: this *Journal*, xiv, p. 207, 1913; *New York Agricultural Experiment Station Technical Bulletins*, No. 26, p. 12, 1912.

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relation between the bases (calcium and magnesium) and the acids (casein and phosphoric acid) is that required, theoretically, to give di-calcium phosphate with a trace of di-magnesium phosphate and the calcium caseinate neutral to phenolphthalein, in which casein is combined with eight equivalents of calcium (casein  $\text{Ca}_4$ ). However, the same analytical figures can with equal correctness be interpreted to prove that the compounds are present as acid caseinate and tri-calcium phosphate.

TABLE III.

*Amounts of calcium and magnesium in insoluble portion of milk.*

COW NO.	STAGE OF LACTATION	CALCIUM			MAGNESIUM		
		Total	Soluble	Insoluble	Total	Soluble	Insoluble
		(11)	(12)	(13)	(14)	(15)	(16)
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1.....	3 dys.	0.1607	0.0734	0.0873	0.0156	0.0142	0.0013
2.....	1 mo.	0.1381	0.0511	0.0870	0.0136	0.0117	0.0019
3.....	1 mo.	0.1362	0.0544	0.0818	0.0180	0.0142	0.0038
3.....	11 mo.	0.1559	0.0534	0.1025	0.0170	0.0156	0.0014
4.....	3 mo.	0.1483	0.0343	0.1140	0.0184	0.0128	0.0056
5.....	3 mo.	0.1396	0.0531	0.0865	0.0156	0.0124	0.0032
5.....	7 mo.	0.1256	0.0454	0.0802	0.0147	0.0134	0.0013
6.....	5 mo.	0.1413	0.0373	0.1040	0.0160	0.0127	0.0033
7.....	6 mo.	0.1464	0.0526	0.0938	0.0144	0.0121	0.0023
7.....	10 mo.	0.1523	0.0450	0.1073	0.0177	0.0127	0.0050
8.....	7 mo.	0.1506	0.0439	0.1062	0.0153	0.0118	0.0035
9.....	8 mo.	0.1503	0.0440	0.1063	0.0171	0.0126	0.0045
10.....	9 mo.	0.1410	0.0543	0.0867	0.0168	0.0141	0.0027
11.....	10 mo.	0.1379	0.0357	0.1022	0.0168	0.0119	0.0049
12.....	11 mo.	0.1659	0.0414	0.1245	0.0191	0.0123	0.0068
13.....	12 mo.	0.2167	0.0669	0.1498	0.0236	0.0163	0.0073

In order to decide which of these sets of compounds is present in milk, we have tried to make a separation of the casein and insoluble phosphates. The results, it will be remembered, are obtained by difference, the milk and serum being analyzed and the composition of the insoluble portion being determined by subtracting the latter results from the former. It seemed desirable to separate milk in large amounts so as to obtain the insoluble portion in quantity sufficient to purify and analyze. This was done in the following manner, several experiments being made.

In the first experiment 400 pounds of milk were run through a cream centrifugal separator eighteen times and the deposit ("separator slime") collecting on the walls of the bowl was removed after the 1st, 6th, 12th, and 18th runs. Each of these deposits was placed in a mortar and triturated with small amounts of 95 per cent alcohol with the gradual addition of more alcohol. A point is reached at which the whole mass becomes jelly-like, after which the addition of more alcohol causes the formation of a

TABLE IV.  
*Amounts of acids and bases expressed as gram equivalents.*

COW NO.	CASEIN AS GRAM EQUIVALENTS OF OCTA-VALENT ACID	INSOLUBLE INOR- GANIC PHOSPHATES AS GRAM EQUIVA- LENTS OF DI-BASIC ACID	SUM OF GRAM EQUIV- ALENTS OF CASEIN AND PHOSPHATES	INSOLUBLE CALCIUM AS GRAM EQUIVA- LENTS	INSOLUBLE MAGNE- SIUM AS GRAM EQUIVALENTS	SUM OF THE INSOL- UBLE CALCIUM AND MAGNESIUM AS GRAM EQUIVA- LENTS	EXCESS OF INSOL- UBLE BASE (+) OR ACID (-) AS GRAM EQUIVALENTS
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1.....	31.3×10 <sup>-4</sup>	13.1×10 <sup>-4</sup>	44.4×10 <sup>-4</sup>	43.6×10 <sup>-4</sup>	1.1×10 <sup>-4</sup>	44.7×10 <sup>-4</sup>	+0.3×10 <sup>-4</sup>
2.....	24.5×10 <sup>-4</sup>	23.1×10 <sup>-4</sup>	47.6×10 <sup>-4</sup>	43.5×10 <sup>-4</sup>	1.6×10 <sup>-4</sup>	45.1×10 <sup>-4</sup>	-2.5×10 <sup>-4</sup>
3.....	25.0×10 <sup>-4</sup>	20.5×10 <sup>-4</sup>	45.5×10 <sup>-4</sup>	40.9×10 <sup>-4</sup>	3.1×10 <sup>-4</sup>	44.0×10 <sup>-4</sup>	-1.5×10 <sup>-4</sup>
3.....	36.8×10 <sup>-4</sup>	18.1×10 <sup>-4</sup>	54.9×10 <sup>-4</sup>	51.2×10 <sup>-4</sup>	1.2×10 <sup>-4</sup>	52.4×10 <sup>-4</sup>	-2.5×10 <sup>-4</sup>
4.....	27.8×10 <sup>-4</sup>	32.0×10 <sup>-4</sup>	59.8×10 <sup>-4</sup>	57.0×10 <sup>-4</sup>	4.6×10 <sup>-4</sup>	61.6×10 <sup>-4</sup>	+1.8×10 <sup>-4</sup>
5.....	25.9×10 <sup>-4</sup>	16.8×10 <sup>-4</sup>	42.7×10 <sup>-4</sup>	43.2×10 <sup>-4</sup>	2.7×10 <sup>-4</sup>	45.9×10 <sup>-4</sup>	+3.2×10 <sup>-4</sup>
5.....	24.3×10 <sup>-4</sup>	22.2×10 <sup>-4</sup>	46.5×10 <sup>-4</sup>	40.1×10 <sup>-4</sup>	1.0×10 <sup>-4</sup>	41.1×10 <sup>-4</sup>	-5.4×10 <sup>-4</sup>
6.....	26.3×10 <sup>-4</sup>	28.7×10 <sup>-4</sup>	55.0×10 <sup>-4</sup>	52.0×10 <sup>-4</sup>	2.7×10 <sup>-4</sup>	54.7×10 <sup>-4</sup>	-0.3×10 <sup>-4</sup>
7.....	30.6×10 <sup>-4</sup>	17.4×10 <sup>-4</sup>	48.0×10 <sup>-4</sup>	46.9×10 <sup>-4</sup>	1.9×10 <sup>-4</sup>	48.8×10 <sup>-4</sup>	+0.8×10 <sup>-4</sup>
7.....	32.0×10 <sup>-4</sup>	26.9×10 <sup>-4</sup>	58.9×10 <sup>-4</sup>	53.7×10 <sup>-4</sup>	4.2×10 <sup>-4</sup>	57.9×10 <sup>-4</sup>	-1.0×10 <sup>-4</sup>
8.....	32.2×10 <sup>-4</sup>	22.3×10 <sup>-4</sup>	54.5×10 <sup>-4</sup>	53.4×10 <sup>-4</sup>	2.9×10 <sup>-4</sup>	56.3×10 <sup>-4</sup>	+1.8×10 <sup>-4</sup>
9.....	31.1×10 <sup>-4</sup>	27.5×10 <sup>-4</sup>	58.6×10 <sup>-4</sup>	53.2×10 <sup>-4</sup>	3.8×10 <sup>-4</sup>	57.0×10 <sup>-4</sup>	-1.6×10 <sup>-4</sup>
10.....	27.9×10 <sup>-4</sup>	17.0×10 <sup>-4</sup>	44.9×10 <sup>-4</sup>	43.4×10 <sup>-4</sup>	2.2×10 <sup>-4</sup>	45.6×10 <sup>-4</sup>	+0.7×10 <sup>-4</sup>
11.....	30.2×10 <sup>-4</sup>	27.0×10 <sup>-4</sup>	57.2×10 <sup>-4</sup>	51.1×10 <sup>-4</sup>	4.1×10 <sup>-4</sup>	55.2×10 <sup>-4</sup>	-2.0×10 <sup>-4</sup>
12.....	28.3×10 <sup>-4</sup>	35.5×10 <sup>-4</sup>	63.8×10 <sup>-4</sup>	62.3×10 <sup>-4</sup>	5.7×10 <sup>-4</sup>	68.0×10 <sup>-4</sup>	+4.2×10 <sup>-4</sup>
13.....	44.7×10 <sup>-4</sup>	32.9×10 <sup>-4</sup>	77.6×10 <sup>-4</sup>	74.9×10 <sup>-4</sup>	6.1×10 <sup>-4</sup>	81.0×10 <sup>-4</sup>	+3.4×10 <sup>-4</sup>

fine flocculent precipitate. (Care must be taken not to add the alcohol too rapidly, because then there is apt to be formed a tough, leathery mass, which can not be handled.) The precipitate is allowed to settle and, after decanting the supernatant liquid, is triturated with several successive portions of 95 per cent alcohol, 99 per cent alcohol, and finally ether. It is then dried at 60°C. for a few hours, after which the drying is completed in a vacuum over sulphuric acid. The analytical results are given in Table V.



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TABLE V.

*Composition of insoluble portion ("separator slime") of milk.*

SAMPLE OF DEPOSIT TAKEN	CASEIN	ASH	TOTAL PHOSPHORUS	PHOSPHORUS IN CASEIN	PHOSPHORUS AS PHOSPHATES	CALCIUM	RATIO OF ORGANIC TO INSOLUBLE INORGANIC PHOSPHORUS Organic P: inorganic P
	per cent	per cent	per cent	per cent	per cent	per cent	
After 1st run.....	86.31	10.43	2.182	0.621	1.561	3.386	1: 2.51
After 6th run.....	90.07	9.35	1.950	0.649	1.301	3.246	1: 2.00
After 12th run.....	90.84	9.53	2.011	0.645	1.366	3.343	1: 2.11
After 18th run.....	91.98	9.62	2.023	0.662	1.361	3.223	1: 2.06

The figures in Table V, obtained by direct analysis of the insoluble deposit or "separator slime," show a striking agreement with results obtained by the indirect method, which is brought out more clearly by expressing the above figures in the form of gram equivalents, as follows:

TABLE VI.

*Amounts of acids and bases expressed as gram equivalents.*

SAMPLE OF DEPOSIT TAKEN	CASEIN AS GRAM EQUIVALENTS OF ACID	PHOSPHATES AS GRAM EQUIVALENTS OF DI-BASIC ACID	SUM OF GRAM EQUIVALENTS OF CASEIN AND PHOSPHATES	GRAM EQUIVALENTS OF CALCIUM
After 1st run.....	$77.7 \times 10^{-3}$	$100.7 \times 10^{-3}$	$178.4 \times 10^{-3}$	$169.3 \times 10^{-3}$
After 6th run.....	$81.1 \times 10^{-3}$	$82.9 \times 10^{-3}$	$164.0 \times 10^{-3}$	$162.3 \times 10^{-3}$
After 12th run.....	$80.8 \times 10^{-3}$	$88.1 \times 10^{-3}$	$168.9 \times 10^{-3}$	$167.2 \times 10^{-3}$
After 18th run.....	$82.8 \times 10^{-3}$	$87.8 \times 10^{-3}$	$170.6 \times 10^{-3}$	$161.2 \times 10^{-3}$

The high percentage of inorganic phosphorus in the deposit from the first run indicates that the phosphates are heavier than the caseinates and could be separated from them if a certain speed were used in running the separator. This point is further shown by the following experiments.

In the first experiment the bowl of a cream-separator was filled with fat-free milk (about 1000 cc.) and was whirled for two hours, at a speed of 5000 revolutions per minute, when the milk was taken out and the "separator slime" which had collected on the bowl was removed and treated with alcohol and ether in the manner already described. The same milk was returned to the separator bowl and again whirled for two hours, when the

deposit was again removed and treated as before. When removed the second time, that is, after four hours of whirling, the milk was nearly as clear as whey, most of the suspended phosphates and casein having been deposited on the walls of the bowl during the whirling. The results of analysis of the "separator slime" deposited after each two hours' whirling are given in Table VII.

These results show that two-thirds of the insoluble inorganic phosphorus was removed during the first two hours of whirling, again indicating that the phosphates are heavier than the casein. The ratio of casein to phosphates is here also shown to be wholly irregular, indicating no definite combination.

Expressing the data in Table VII in the form of gram equivalents, we have the figures contained in Table VIII.

TABLE VII.  
*Composition of insoluble portion of milk deposited at different intervals.*

"SLIME" FORMED BY WHIRLING FOR 2 HR. PERIODS	CASEIN	ASH	TOTAL PHOS- PHORUS	PHOS- PHORUS IN CASEIN	PHOS- PHORUS AS PHOS- PHATES	CALCIUM	RATIO OF ORGANIC TO INSOLUBLE INORGANIC PHOS- PHORUS
							Organic P: inorganic P
	per cent	per cent	per cent	per cent	per cent	per cent	
1st 2 hrs.	90.68	9.32	1.909	0.653	1.256	3.090	1:1.92
2d 2 hrs.	91.12	8.88	1.437	0.656	0.781	2.691	1:1.19

An examination of these figures shows that there is the same balance between the acids (casein and phosphoric acid) and the bases (calcium and magnesium) in the two separate deposits, even when the inorganic phosphorus is so unevenly distributed between them, which furnishes proof for two points: (1) The inorganic phosphorus must be in the form of neutral calcium phosphate ( $\text{CaHPO}_4$ ); for otherwise the balance between bases and acids would be altered, acid calcium phosphate ( $\text{CaH}_4\text{P}_2\text{O}_8$ ) giving an excess of acid, and tri-calcium phosphate ( $\text{Ca}_3\text{P}_2\text{O}_8$ ) an excess of base in the "slime" deposited in the first whirling. (2) If the phosphates were in combination with the casein we should expect to find the ratio between the organic phosphorus and the inorganic phosphorus the same in both deposits, but instead of uniformity we find the ratio showing as wide a variation as 1:1.92 and 1:1.19 in the two cases.

In the second experiment further evidence is furnished, showing that neutral calcium phosphate ( $\text{CaHPO}_4$ ) is a normal constituent of milk.

Four 500 cc. bottles were filled with separator skim-milk to which some formaldehyde had been added, and after standing at room temperature for four days, were whirled in a Bausch and Lomb precision centrifugal machine for thirty minutes at a speed of 1200 revolutions per minute. A sediment was deposited, which after purification by treatment with alcohol and ether, as previously described, weighed 0.4 gm.

Analysis of this gave the following results: casein, 20.78 per cent; total phosphorus, 18.38 per cent; phosphorus combined with casein, 0.15 per cent; phosphorus combined as phosphates, 18.23 per cent; calcium, 22.79 per cent; ratio of organic phosphorus to inorganic phosphorus, 1:121; casein as gram equivalents of acid,

TABLE VIII.

*Amounts of acids and bases expressed as gram equivalents.*

	CASEIN AS GRAM EQUIVALENTS OF ACID	PHOSPHATES AS GRAM EQUIVALENTS OF DI-BASIC ACID	SUM OF GRAM EQUIVALENTS OF CASEIN AND PHOSPHATES	GRAM EQUIVALENTS OF CALCIUM
1st deposit.....	$81.6 \times 10^{-3}$	$81.1 \times 10^{-3}$	$162.7 \times 10^{-3}$	$154.5 \times 10^{-3}$
2d deposit.....	$82.0 \times 10^{-3}$	$50.4 \times 10^{-3}$	$132.4 \times 10^{-3}$	$134.6 \times 10^{-3}$

$18.7 \times 10^{-3}$ ; phosphates as gram equivalents of di-basic acid,  $1175 \times 10^{-3}$ ; sum of casein and phosphates as gram equivalents of acid,  $1194 \times 10^{-3}$ ; gram equivalents of calcium,  $1140 \times 10^{-3}$ .

In these figures we again find the same balance between bases and acids, which can mean only that the phosphate compound deposited is di-calcium phosphate ( $\text{CaHPO}_4$ ). The degree of centrifugal force developed was sufficient to throw out a relatively large amount of di-calcium phosphate, but not powerful enough to throw out very much casein, thus serving as a means of effecting a nearly complete separation of these two constituents.

Babcock<sup>5</sup> whirled skim-milk in a separator for several hours, removing portions from time to time for analysis and finally determined the amounts of casein, calcium, and phosphorus in

<sup>5</sup> S. M. Babcock: *Wisconsin Agricultural Experiment Station, 12th Annual Report*, p. 93, 1895.

the deposited "slime." While the experiments were preliminary in character and the results not sufficient to base permanent conclusions on, they tended to show that the casein and phosphates were not in combination. From the analytical results showing the relation of calcium to phosphorus, the conclusion was drawn that tri-calcium phosphate is the compound present in milk. The figures for calcium and phosphorus were based upon the total amounts contained in the deposit and no allowance was made for the calcium in combination with casein and the phosphorus of the casein. This fact accounts for the difference between the results reported by him and the conclusions reached by us. A recalculation of his data, after deducting the amounts of calcium and phosphorus combined with casein, gives figures that correspond to the composition of  $\text{CaHPO}_4$  and not  $\text{Ca}_3\text{P}_2\text{O}_8$ , thus confirming the results of our work.

*Acidity of milk and milk serum.*

Both fresh milk and the serum from fresh milk show a slight acid reaction to phenolphthalein. This has been believed to be due to casein or acid phosphates in the milk or to both. The fact that fresh milk and its serum are strongly alkaline to methyl orange indicates that the acidity is due to acid phosphates, though it does not necessarily show that acid caseinates are not also responsible for some of the acidity. The results of our work given in the preceding pages furnish aid in determining to what compounds in milk the acid reaction to phenolphthalein is due.

A 1000 cc. sample of milk was obtained from each of eight cows immediately after milking, and chloroform (50 cc.) was added to this at once. The acidity of the milk and of the serum was determined after treatment with neutral potassium oxalate according to the method of Van Slyke and Bosworth.<sup>6</sup> The results are given in Table IX.

These figures show that the acidity of fresh milk is the same as that of its serum, which means that the constituents of the milk causing acidity are soluble constituents contained in the serum. Since the serum contains phosphates in amounts sufficient to furnish two to four times the acid phosphates required

<sup>6</sup>L. L. Van Slyke and A. W. Bosworth: this *Journal*, xix, p. 73, 1914.

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to account for the acidity, and since, moreover, no other acid constituents of the milk serum are present in more than minute quantities, and are wholly insufficient to cause the observed degree of acidity, it appears a reasonable conclusion that the acidity of fresh milk is due to soluble acid phosphates. This conclusion is further strengthened by the results given in the preceding pages which go to show conclusively that the insoluble constituents of fresh milk are neutral in reaction, consisting largely or wholly of neutral calcium caseinate (casein  $\text{Ca}_4$ ), neutral dicalcium phosphate ( $\text{CaHPO}_4$ ), and fat.

TABLE IX.  
*Acidity of milk and milk serum.*

NO. OF SAMPLE	NO. OF CC. OF $\frac{N}{10}$ ALEALI REQUIRED TO NEUTRALIZE 100 CC. OF	
	Milk	Milk serum
1.....	4.8	5.0
2.....	6.2	6.2
3.....	4.2	4.2
4.....	6.0	5.8
5.....	6.4	6.4
6.....	4.4	4.4
7.....	7.0	6.8
8.....	6.6	6.4

### *Compounds of milk.*

It is difficult to learn what are the individual forms or compounds in which the salts exist in milk. Attempts have been made to determine this by inference based on analytical results. In view of the data presented in the preceding pages, taken together with many other analytical data worked out by us, we suggest the following statement as representing in some respects more closely than previous ones the facts corresponding to our present knowledge of the principal constituents of milk. The amounts are based on milk of average composition.<sup>7</sup>

<sup>7</sup> L. L. Van Slyke: *Modern Methods of Testing Milk and Milk Products*, New York, 1912.

	<i>Per cent</i>
Fat.....	3.90
Milk sugar.....	4.90
Proteins combined with calcium.....	3.20
Di-calcium phosphate ( $\text{CaHPO}_4$ ).....	0.175
Calcium chloride ( $\text{CaCl}_2$ ).....	0.119
Mono-magnesium phosphate ( $\text{MgH}_2\text{P}_2\text{O}_7$ ).....	0.103
Sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ).....	0.222
Potassium citrate ( $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$ ).....	0.052
Di-potassium phosphate ( $\text{K}_2\text{HPO}_4$ ).....	0.230
Total solids.....	12.901

## CONCLUSIONS.

1. Milk contains two general classes of compounds, those in true solution and those in suspension, or insoluble. These two portions can be separated for study by filtering the milk through a porous earthenware filter like the Pasteur-Chamberland filtering tube.

2. Serum prepared from fresh milk is yellow with a faint greenish tinge and slight opalescence. The following constituents of milk are wholly in solution in the milk serum: sugar, citric acid, potassium, sodium, and chlorine. The following are partly in solution and partly in suspension: albumin, inorganic phosphates, calcium, magnesium. Albumin in fresh milk appears to be adsorbed to a considerable extent by casein and therefore only a part of it appears in the serum. In serum from sour milk and milk to which formaldehyde has been added, nearly all of the albumin appears in the serum.

3. The insoluble portion of milk separated by filtration through Pasteur-Chamberland filtering tubes is grayish to greenish white in color, of a glistening, slime-like appearance, and of gelatinous consistency. When shaken with water it goes into suspension, forming a mixture having the opaque, white appearance of milk. Such a suspension is neutral to phenolphthalein. When purified, the insoluble portion consists of neutral calcium caseinate (casein  $\text{Ca}_4$ ) and neutral di-calcium phosphate ( $\text{CaHPO}_4$ ). The casein and di-calcium phosphate are not in combination, as shown by a study of sixteen samples of milk from thirteen individual cows, and also by a study of the deposit or "separator slime" formed by whirling milk in a cream-separator. By treating fresh milk with for-

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maldehyde and whirling in a centrifugal machine under specified conditions, it is possible to effect a nearly complete separation of phosphates from casein.

4. Both fresh milk and the serum from fresh milk show a slight acid reaction to phenolphthalein, but are strongly alkaline to methyl orange, indicating that acidity is due, in part at least, to acid phosphates. In eight samples of fresh milk the acidity of the milk and of the milk serum was determined after treatment with neutral potassium oxalate. The results show that the acidity of the whole milk is the same as that of the serum, and that, therefore, the constituents of the serum are responsible for the acidity of milk. There is every reason to believe that the phosphates of the serum cause the observed acidity.

5. The data presented, taken together with results of other work, furnish a basis for suggesting an arrangement of the individual compounds contained in milk, especially including the salts.

**RESEARCHES ON PURINES. XVI.<sup>1</sup>**  
**ON THE ISOMERIC MONOMETHYL DERIVATIVES OF 2-METHYL-  
MERCAPTO-4-AMINO-6-OXYPYRIMIDINE. ON 1-METHYL-  
2-METHYLMERCAPTO-6,8-DIOXYPURINE.**

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When 2-thio-4-amino-6-oxypyrimidine (I)<sup>2</sup> is alkylated by means of methyl iodide or dimethyl sulphate, position 2, which contains the sulphur atom, is the first to be attacked, and 2-methylmercapto-4-amino-6-oxypyrimidine (III)<sup>3</sup> is formed. That the methyl group has become attached to sulphur is shown by the fact that methylmercaptan is evolved when the pyrimidine is boiled with concentrated hydrochloric acid.

When 2-methylmercapto-4-amino-6-oxypyrimidine (III) was methylated with dimethyl sulphate, a second methyl group was attached to the pyrimidine ring and two isomeric methyl derivatives were formed. One of these was produced only in small quantity, about 20 per cent of the calculated yield. This compound was very soluble in ether and melted at 144°C. Its isomer, which was the chief product of the reaction, was not soluble in ether and melted at 255°C.

It seemed probable that 2-methylmercapto-4-amino-6-oxypyrimidine (III) would methylate on the nitrogen atom in position 1 or on the oxygen atom in position 6, forming either 1-methyl-2-methylmercapto-4-amino-6-oxypyrimidine (VI) or 2-methylmercapto-4-amino-6-methoxypyrimidine (IV). The compound which melted at 144°C. and which was soluble in ether proved to be the methoxy derivative (IV). This was shown by pre-

<sup>1</sup> C. O. Johns and B. M. Hendrix: this *Journal*, xix, p. 25, 1914. The present investigation was aided by a grant from the Bache fund.

<sup>2</sup> W. Traube: *Ann. d. Chem.*, cccxxi, p. 71, 1904.

<sup>3</sup> Johnson and Johns: *Am. Chem. Jour.*, xxxiv, p. 181, 1905; C. O. Johns and E. J. Baumann: this *Journal*, xiv, p. 384, 1913.



paring it by the action of sodium methoxide on 2-methylmercapto-4-amino-6-chlorpyrimidine (V).<sup>4</sup> These substances reacted smoothly to give 2-methylmercapto-4-amino-6-methoxypyrimidine (IV), which was found to be identical with the compound which melted at 144°C.

It is, therefore, probable that the compound which melted at 255°C. was 1-methyl-2-methylmercapto-4-amino-6-oxypyrimidine (VI). This view is supported by the fact that in the case of all the pyrimidines which we have methylated and which contained an amino group in positions 4 or 6, the methyl group has attached itself to the nitrogen atom farthest away from the amino group. Thus, pyrimidines containing an amino group in position 6 have methylated in position 3, or what might be termed the position "para" to the amino group.<sup>5</sup> If this rule holds, 2-methylmercapto-4-amino-6-oxypyrimidine (III) would methylate in position 1, and the methyl derivative would be 1-methyl-2-methylmercapto-4-amino-6-oxypyrimidine (VI). This compound was obtained in this laboratory some years ago as a by-product in methylating 2-thio-4-amino-6-oxypyrimidine (I) in the presence of an excess of sodium ethylate and methyl iodide. We supposed at that time that it was a methoxy derivative and suggested that its formula was 2-methylmercapto-2-amino-6-methoxypyrimidine (IV).<sup>6</sup> The results of the present investigation show that we were mistaken, because the compound melted at 255°C. and was identical with 1-methyl-2-methylmercapto-4-amino-6-oxypyrimidine (VI).

Since two isomeric methyl derivatives are formed when 2-methylmercapto-4-amino-6-oxypyrimidine (III) is methylated, it is probable that this compound exists in two tautomeric forms. These two forms may be represented by formulas II and III. On methylation the former would give a 6-methoxy derivative and the latter a 1-methyl compound. The supposition that it exists in the enol form is supported by the fact that it gives 2-methylmercapto-4-amino-6-chlorpyrimidine (V)<sup>7</sup> when boiled with phos-

<sup>4</sup> Johnson and Johns: *Am. Chem. Jour.*, xxxiv, p. 183, 1905.

<sup>5</sup> Johns: this *Journal*, xi, p. 75, 1912; Johns and Baumann: *ibid.*, xvi, p. 137, 1913.

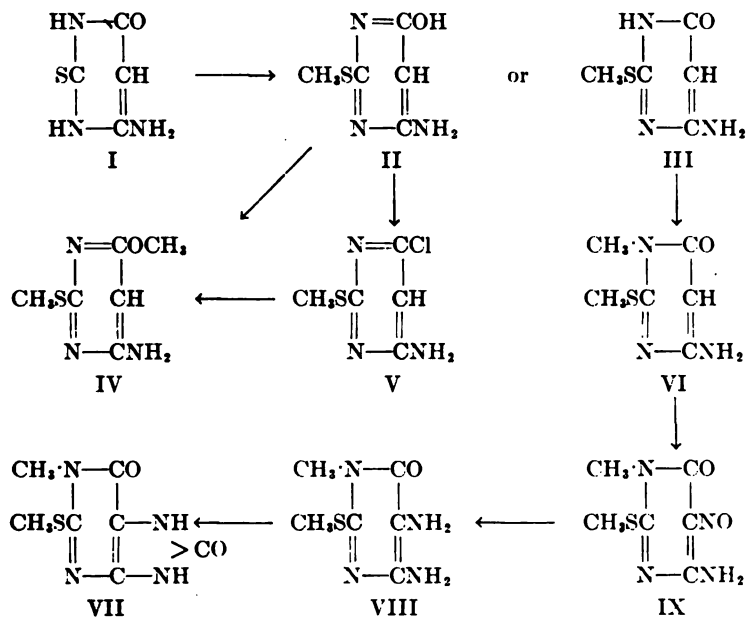
<sup>6</sup> Johnson and Johns: *Am. Chem. Jour.*, xxxiv, p. 182, 1905.

<sup>7</sup> Johnson and Johns: *loc. cit.*

phorus oxychloride, a reaction in which an OH group must take part. It is also noteworthy that the yield of the chloride is less than 50 per cent of the calculated quantity even when a very great excess of phosphorus oxychloride is used, and that a large portion of the 2-methylmercapto-4-amino-6-oxypyrimidine (III) is recovered unaltered.

When 1-methyl-2-methylmercapto-4-amino-6-oxypyrimidine (VI) was treated with nitrous acid, an almost quantitative yield of 1-methyl-2-methylmercapto-4-amino-5-nitroso-6-oxypyrimidine (IX) was obtained. By means of ammonium sulphide this compound was reduced to 1-methyl-2-methylmercapto-4,5-diamino-6-oxypyrimidine (VIII). When the latter compound was heated with urea, an almost quantitative yield of 1-methyl-2-methylmercapto-6,8-dioxypurine (VII) was obtained.

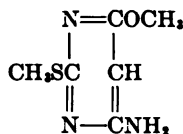
These researches will be continued.



## EXPERIMENTAL PART.

*Methylation of 2-methylmercapto-4-amino-6-oxypyrimidine.*

Ten grams of 2-methylmercapto-4-amino-6-oxypyrimidine<sup>8</sup> were dissolved in 65 cc. of normal potassium hydroxide solution, and 9 grams of dimethyl sulphate were added gradually while the solution was agitated by frequent shaking. A white, crystalline precipitate began to appear almost immediately, and this soon became very bulky. As soon as the solution became acid to litmus the crystals were filtered off by suction. The filtrate was neutralized with sodium hydroxide and evaporated to dryness. The residue was washed with cold water, the solid was filtered off and added to the crystals already obtained. The combined solids were then triturated with dilute ammonia to dissolve any unaltered 2-methylmercapto-4-amino-6-oxypyrimidine, a small quantity of which was found to be present. That part of the residue which was not soluble in ammonia consisted of two compounds which differed widely as to their melting points and solubility in ether. The compound having the lower melting point was very soluble in ether, while the one with the higher melting point was almost insoluble in this solvent. Ether, therefore, served as a means of separating these compounds from each other.

*2-Methylmercapto-4-amino-6-methoxypyrimidine.*

The ether extract which was obtained in the above experiment was evaporated to dryness. A white crystalline residue remained. This was recrystallized from dilute alcohol by dissolving it in about 50 per cent alcohol and concentrating the solution until crystals appeared on cooling. In this manner a beautiful, crystalline substance was obtained. This was very soluble in alcohol, ether, or benzene. It was moderately soluble in hot and difficultly soluble in cold water. The crystals sublimed slowly when heated

<sup>8</sup> Johns and Baumann: *loc. cit.*

to about 90°C. Both the crystals and their sublimate melted to an oil at 144°C. The yield was 20 per cent of the calculated quantity.

	Calculated for $C_6H_7ON_3S$ :	Found:
N.....	24.57	24.69

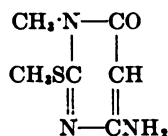
The structure of the compound just described was ascertained in the following manner:

5.8 grams of 2-methylmercapto-4-amino-6-chlorpyrimidine<sup>9</sup> were dissolved in 50 cc. of methyl alcohol to which 1.6 grams of metallic sodium had previously been added. This mixture was heated under a reflux condenser on a water bath for an hour. Sodium chloride was deposited during the process of heating. The alcohol was removed by evaporation on a steam bath. The residue was stirred with water and this mixture was acidified with hydrochloric acid. This treatment left a white residue which was recrystallized from dilute alcohol. The crystals melted at 143°C. and had all of the properties of the compound which we had obtained from the ether extract, as described above.

	Calculated for $C_6H_7ON_3S$ :	Found:
N.....	24.57	24.38

Samples of the compound obtained from the ether extract and by the action of sodium methoxide on 2-methylmercapto-4-amino-6-chlorpyrimidine were mixed and the mixture was found to melt at 144°C. Hence the two were identical.

*1-Methyl-2-methylmercapto-4-amino-6-oxypyrimidine.*



This was the chief product of the reaction between dimethyl sulphate and 2-methylmercapto-4-amino-6-oxypyrimidine, under the conditions described above, and was the portion not soluble in ether. After dissolving out the 2-methylmercapto-4-amino-6-

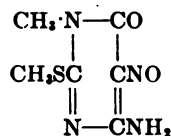
<sup>9</sup> Johnson and Johns: *loc. cit.*

methoxypyrimidine by means of ether and filtering, the solid residue was recrystallized from alcohol. It separated in the form of slender prisms. These melted to an oil at 255°C. They were moderately soluble in hot water or alcohol, but almost insoluble in ether or benzene. The yield was 60 per cent of the calculated quantity.

	Calculated for C <sub>6</sub> H <sub>7</sub> ON <sub>2</sub> S:	Found:
N.....	24.57	24.71

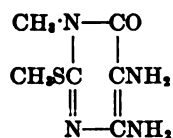
This substance agreed in all respects with the compound which had previously been obtained in this laboratory as a by-product by the action of an excess of methyl iodide and sodium ethylate on 2-thio-4-amino-6-oxypyrimidine. We assumed at that time that this compound was 2-methylmercapto-4-amino-6-methoxypyrimidine. The results of the present investigation indicate that it was 1-methyl-2-methylmercapto-4-amino-6-oxypyrimidine.

*1-Methyl-2-methylmercapto-4-amino-5-nitroso-6-oxypyrimidine.*



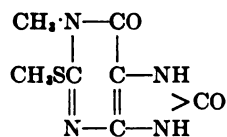
4.8 grams of 1-methyl-2-methylmercapto-4-amino-6-oxypyrimidine were suspended in an excess of dilute hydrochloric acid and a solution of 2.5 grams of sodium nitrite in a little water was added gradually. The suspended material assumed a deep blue color and became crystalline and very bulky. The reaction was complete in about fifteen minutes, after which the solid material was filtered off and washed with cold water. The portion used for analysis was recrystallized from water and was so obtained in the form of slender prisms of a deep blue color. These melted at 235°C. They were sparingly soluble in hot water or alcohol and almost insoluble in benzene or ether. The aqueous and alcoholic solutions possessed the deep blue color of an ammoniacal copper sulphate solution. The yield was almost quantitative.

	Calculated for C <sub>6</sub> H <sub>7</sub> O <sub>2</sub> N <sub>2</sub> S:	Found:
N.....	28.00	27.94

*1-Methyl-2-methylmercapto-4,5-diamino-6-oxypyrimidine.*

5.2 grams of 1-methyl-2-methylmercapto-4-amino-5-nitroso-6-oxypyrimidine were finely pulverized and suspended in 150 cc. of water. The nitroso compound was reduced by adding ammonium sulphide gradually until an excess seemed to be present. The reduction proceeded very rapidly and the nitroso compound all dissolved, sulphur being precipitated. After a half hour the reaction seemed complete and the sulphur was filtered off. The filtrate was concentrated to about 50 cc. to remove more sulphur and drive off the excess of ammonium sulphide. On filtering again and cooling, a crystalline precipitate was obtained. This was recrystallized from water and it separated in the form of acicular prisms, which melted at 212°C. They were moderately soluble in hot water or alcohol, but almost insoluble in ether or benzene. The yield was only about 50 per cent of the theoretical quantity.

	Calculated for $\text{C}_6\text{H}_8\text{ON}_4\text{S}$ :	Found:
N .....	30.11	30.06

*1-Methyl-2-methylmercapto-6,8-dioxypurine.*

1-Methyl-2-methylmercapto-4,5-diamino-6-oxypyrimidine was heated with an equal weight of urea in an oil bath at 160–170°C. for an hour. The mixture melted and ammonia was evolved, then it solidified to a hard cake. This material was pulverized and washed with cold water. The residue was dissolved in dilute ammonia to which a little sodium hydroxide was added. On filtering and acidifying with acetic acid, a powder was precipitated.

This was composed of extremely small crystals. These decomposed above 300°C. without melting. They were slightly soluble in hot water or alcohol but did not dissolve in ether or benzene. They did not give the murexide reaction. The yield was almost quantitative.

	Calculated for $C_7H_5O_2N_4S$ :	Found:
N.....	26.41	26.46

## LIPINS IN NUTRITION.

By C. G. MACARTHUR AND C. L. LUCKETT.

(From the Department of Biological Chemistry, University of Illinois, Urbana.)

(Received for publication, December 19, 1914.)

There is considerable difference of opinion as to the necessity of certain lipin-like substances for maintenance and growth. The earlier investigators did not consider the lipins as an essential part of a food. But recently several important papers<sup>1</sup> have appeared indicating the essential presence in complete rations of a substance with fat-like solubilities. There is a rather wide range of opinion as to the nature of this material. An examination of the articles just referred to will show that the subject has been approached from several angles; and though the fats, cholesterol, the phosphatids, and the cerebrosides have at one time or another been suspected of being the essential substance, they are not now generally supposed to be so.

One of two objections can be offered to most of the previous investigations on this subject. In some the food used did not consist of pure, definite substances; all the difficulties encountered in an unknown mixture apply here. In others the synthetic food was probably not lipoid-free. Ether does not remove all the lipoids; even cold alcohol fails to extract them completely; hot alcohol or a hot benzene and alcohol mixture seems to be necessary.

This investigation was carried out with the hope that by mak-

<sup>1</sup> W. Stepp: *Biochem. Ztschr.*, xxii, p. 452, 1909; *Ztschr. f. Biol.*, lvii, p. 135, 1912; lxii, p. 405, 1913.

L. B. Mendel and T. B. Osborne: this *Journal*, xii, p. 81, 1912; xiii, p. 233, 1912-13; xvi, p. 423, 1913-14.

E. V. McCollum, J. G. Halpin, and A. H. Drescher: this *Journal*, xiii, p. 219, 1912-13.

E. V. McCollum and M. Davis: this *Journal*, xv, p. 167, 1913.

C. Funk: *Jour. Physiol.*, xliii, p. 395, 1911-12; xliv, p. 50, 1912.

E. A. Cooper: *Biochem. Jour.*, viii, p. 347, 1914.

F. G. Hopkins: *Jour. Physiol.*, xliv, p. 425, 1912.



ing the food used largely of definite substances and by thoroughly extracting the substances fed, when contamination with lipins was at all possible, some clearer ideas of this uncertain substance might be obtained.

*Preparation of food.*

The lard was of the best quality and gave no nitrogen test. The starch and lactose were phosphorus- and nitrogen-free.

The ordinary casein, referred to as casein (1), was refluxed with alcohol for three two day periods, and was filtered and pressed after each period. It was finally washed with ether and dried. In the later experiments the purest casein (2) (Hammarsten) was fed.

The part of the ration most difficult to free from lipins is the salts. Salt mixture<sup>2</sup> (i) was not adequate, although used as

SALT MIXTURE (i).

	gm.
Calcium phosphate.....	10
Potassium hydrogen phosphate.....	37
Sodium chloride.....	20
Sodium citrate.....	15
Magnesium citrate.....	8
Calcium lactate.....	8
Iron citrate.....	2

supplementary food. A more successful combination of salts<sup>3</sup> is mixture (ii). Probably the most satisfactory purely synthetic

SALT MIXTURE (ii).

	gm.
Hydrochloric acid.....	12.75
Phosphoric acid.....	10.32
Citric acid.....	10.10
Sulphuric acid.....	0.92
Calcium carbonate.....	13.84
Magnesium carbonate.....	2.42
Potassium carbonate.....	14.13
Sodium carbonate.....	14.04
Iron citrate.....	0.634

salt mixture (iii) contains these ingredients:<sup>4</sup>

<sup>2</sup> F. Röhmman: *Jahresb. f. Thier-Chem.*, xxxviii, p. 659, 1909.

<sup>3</sup> Osborne and Mendel: *Ztschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

<sup>4</sup> McCollum (unpublished).

## SALT MIXTURE (iii).

	gm.
Sodium chloride.....	0.146
Magnesium sulphate.....	0.225
Sodium dihydrogen phosphate.....	0.293
Potassium monohydrogen phosphate.....	0.805
Calcium tetrahydrogen phosphate.....	0.456
Iron lactate (Merck).....	0.100
2.4 gm. of the above and 1.3 gm. of calcium lactate were used with each 100 gm. of ration.	

In some of the experiments the salts, carbohydrate, and part of the protein were supplied by milk powder<sup>5</sup> (iv), which was treated like the casein, with alcohol and ether, because it had been indicated in preliminary experiments that the milk powder contained the substances to be investigated.

However, protein-free milk<sup>6</sup> (v) is the surest source of salts. They were prepared in the usual way, then extracted with hot alcohol and ether alternately, twice with each solvent. After the first experiments it was found that three one day periods of extraction with a mixture of benzene and absolute alcohol (60 per cent benzene, 40 per cent alcohol) were more efficient in removing fats and less liable to remove necessary salts. After this treatment the benzene was removed from the residue by washing thoroughly with ether. Protein-free milk contains lactose.

To obtain lipoids the yolks of eggs were spread on plates and dried in an air drier at room temperature. The dry yolk was extracted with ether and the *egg cephalin* (a) precipitated with alcohol. The filtrate was evaporated carefully to dryness at low temperature, taken up in ether, and the *egg lecithin* (b) precipitated by acetone. The filtrate was slowly evaporated and labeled the *third fraction* (c). This third fraction is almost entirely ordinary fats and cholesterol. Some mice were fed on unseparated *alcohol and ether extract* (d), which consists of cephalin, lecithin, small amounts of other phosphatids, fats, cholesterol, cerebroside-like substances, and an unknown compound or compounds. In order to study more completely the alcohol extract, the dried

<sup>5</sup> Merrell Soule's skimmed milk powder.

<sup>6</sup> Osborne and Mendel: *Carnegie Institution of Washington Publications*, No. 156, pt. ii, 1911.

yolks were in some cases treated with hot alcohol and allowed to cool. This gave a *cold alcohol-soluble part* (e), consisting largely of lecithin, cholesterol, fats, some cerebrosides, and unknown material, and it gave also a *hot alcohol-soluble part* (f), consisting of cerebrosides, cephalin, and some unknown substances.

Another portion of dried egg was first extracted with cold alcohol. This gave the *cold alcohol extract* (g). In this extract are cephalin, lecithin, fats, cholesterol, cerebrosides, and unknown substances. A portion of this extract was evaporated in vacuum to dryness and the residue treated with ether. This gives the *ether-soluble portion* (h), which of course contains the cephalin, lecithin, fats, and cholesterol. The egg tissue was then treated with hot alcohol. This is the *hot alcohol extract* (i), of cerebrosides, cephalin, and unknown material.

#### EXPERIMENTS AND DISCUSSION.

The white mice used in the work were kept at a rather uniform temperature of 75°–80°F. in a moderately well lighted basement room. The usual precautions as to cleanliness were observed. The mice were supplied with water by the bottle hanging-drop method. They were fed regularly, usually every other day. The amount of food eaten was originally recorded, but later was only irregularly noted, because it was found that the amounts of food eaten indicated no points that the records of weight did not show, and were difficult to obtain accurately, owing to the scattering of the food by the mice.

The question of palatability may enter into this work, but probably a food is palatable if it is complete and satisfies all physiological needs, while an incomplete food will not be so readily eaten. Whenever any food mixture was given to a mouse accustomed to an ordinary diet of corn, dog biscuit, sunflower-seeds, carrots, and meat, there was a short period of slower growth, or even no growth, followed by nearly normal growth if the food mixture was complete.

For purposes of comparison, the rate of normal growth of a mouse under the conditions of these experiments is given in the following table:

## EXPERIMENT 40.

FOOD	DAYS	WEIGHT
		<i>gm.</i>
Crushed corn.....	1	13.5
Sunflower-seed.....	12	17.
Meat.....	19	21.
Carrots.....	26	22.
Dog biscuit.....	42	22.2

To see if the fats themselves are indispensable, mice were fed lipid-free food, to which was added olive oil, or lard, or butter.

## EXPERIMENT 14.

FOOD		DAYS	WEIGHT
	<i>gm.</i>		<i>gm.</i>
Ether and alcohol extracted casein (1) .....	18.	1	12.7
Ether and alcohol extracted milk powder (iv) ..	22.	10	12.5
Corn starch.....	23.8	20	12.5
Lactose.....	9.2	27	12.9
Salt mixture (i).....	1.	34	11.5
Lard.....	25.	41*	11.3
*Alcohol and ether extracted egg yolk (d) added		48	12.1
on 41st day .....	3.	55	13.3
		62	14.3
		69	13.7
		79	14.
		83	14.
		90	14.8
		97	15.6
		104	15.7
		111	15.9
		132	18.

## EXPERIMENT 15.

FOOD	gm.	DAYS	WEIGHT	
			Male	Female
			gm.	gm.
Ether and alcohol extracted casein (1) ...	18.	1	17.	15.7
Ether and alcohol extracted milk powder (iv).....	22.	10	17.9	17.5
Corn starch.....	23.8	17	17.9	16.2
Lactose.....	9.2	24	15.9	14.9
Salt mixture (i).....	2.	31	14.	13.7
Creamery butter.....	25.	38	11.2	11.
		42	10.2	9.9
		46	Died.	

## EXPERIMENT 16.

FOOD	gm.	DAYS	WEIGHT
			Male
			gm.
Ether and alcohol extracted casein (1) .....	18.	1	14.3
Ether and alcohol extracted milk powder (iv).....	22.	10	12.5
Corn starch.....	23.8	17	11.3
Lactose.....	9.2	24	9.5
Salt mixture (i).....	1.	31	8.
Olive oil.....	25.	38	7.8
		42	6.7
			Died.

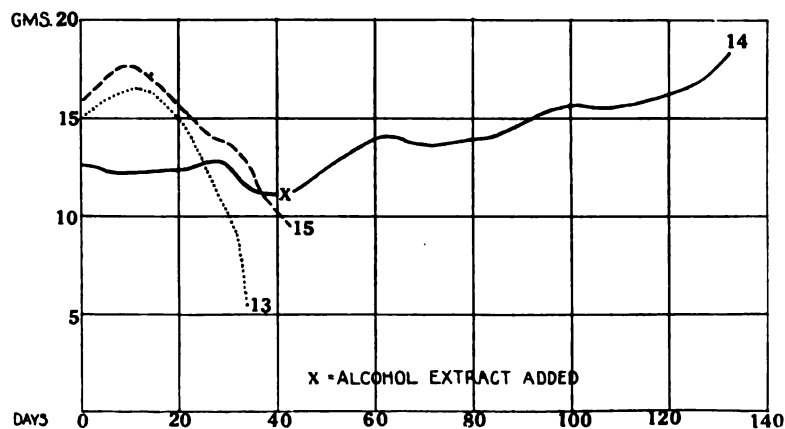


FIG. 1.

These data strongly suggest that olive oil, lard, or butter, when added to the ration, do not make the food a complete one for mice. It is possible that the difference of these results from those of other workers who have found butter to contain the essential material can be attributed to the more complete removal in experiments here given of lipoid-like substances from the rest of the food. Possibly, however, white rats do not require the same substances in their food that white mice require.

It is to be noted in Experiment 14 that the fast failing animal using lard as the only fat in its food very quickly resumed its growth on the addition of an alcohol and ether extract (d) of egg yolk.

To find out what portion of the alcohol-ether extract was responsible for this growth, the various separations previously described were carried out and the products fed.

## EXPERIMENT 29.

FOOD		DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Alcohol and ether extracted casein (1) . . . .	18.	1	10.	11.
Extracted protein-free milk (v) . . . . .	29.5	15	13.2	14.8
Starch . . . . .	23.5	19	13.4	15.
Lard . . . . .	25.	26	15.2	17.2
No lipoids . . . . .		33	13.5	16.9
		40	13.	15.1
		47	12.	14.2
		60	Died.	

## EXPERIMENT 23.

FOOD		DAYS	WEIGHT	
			Male	Female
	<i>gm.</i>		<i>gm.</i>	<i>gm.</i>
Ether and alcohol extracted casein (1)...	18.	1	11.	15.1
Extracted protein-free milk (v).....	29.5	8	8.	12.
Starch.....	26.5	15	8.	14.5
Lard.....	26.	22	8.1	16.4
Lecithin (b).....	5.	29	6.7	15.5
		39	5.5	14.3
		43	Died.	14.
		50		14.
		57		13.6
		64		10.5
				Died.

## EXPERIMENT 28.

FOOD		DAYS	WEIGHT	
			Male	Female
	<i>gm.</i>		<i>gm.</i>	<i>gm.</i>
Ether and alcohol extracted casein (1)...	18.	1	15.	15.6
Salt mixture (iii).....	3.7	16	16.	17.
Starch.....	28.3	20	15.3	16.
Lard.....	25.	27	16.7	14.9
Lactose.....	20.	34	16.	15.
Cephalin (a).....	3.	43	15.5	16.
		50	13.4	16.

## EXPERIMENT 39.

FOOD		DAYS	WEIGHT
	<i>gm.</i>		<i>gm.</i>
Ether and alcohol extracted casein (1).....	18.	1	13.2
Extracted protein-free milk (v).....	29.5	12	14.
Starch.....	26.5	19	14.3
Lard.....	26.	26	15.
Third fraction (c).....	5.	42	Died.

## EXPERIMENT 26.

FOOD		DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Ether and alcohol extracted casein (1)...	18.	1	15.6	17.
Salt mixture (iii).....	3.7	5	17.8	17.3
Starch.....	28.3	12	19.6	17.9
Lard.....	25.	19	20.9	18.
Lactose.....	20.	29	20.4	24.
Dried egg yolk.....	10.	33	20.5	24.1
				Young.
		40	23.7	20.5
		47	23.2	19.8
		54	21.4	21.2
		61	21.	20.9

## EXPERIMENT 27.

FOOD		DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Ether and alcohol extracted casein (1)...	18.	1	17.4	15.
Salt mixture (iii).....	3.7	16	20.6	17.
Starch.....	28.3	20	20.5	17.4
Lard.....	25.	27	19.5	18.8
Lactose.....	20.	34	18.5	16.9
Hot alcohol extract of egg (e and f).....	3.	41	19.2	18.1
		48	20.8	19.4
		68	22.0	16.1

## EXPERIMENT 36.

FOOD		DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Ether and alcohol extracted casein (1)...	18.	1	14.3	9.4
Extracted protein-free milk (v).....	29.5	8	(10.5)	14.
Starch.....	23.5	12	14.9	14.5
Lard.....	25.	19	18.5	15.4
Hot alcohol-soluble part (f).....	5.	26	19.7	
		37	20.5	
		40	21.5	16.6
		60	20.2	



## EXPERIMENT 37.

FOOD	gm.	DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Ether and alcohol extracted casein (1)...	18.	1	15.	17.
Extracted protein-free milk (v) .....	29.5	8	16.	17.2
Starch .....	23.5	12	15.9	17.6
Lard .....	25.	19	18.	21.
Cold alcohol-soluble part (e) .....	5.	26	18.	24.
				Young.
		33	17.4	18.
		40	18.2	20.9
		60	18.4	21.1

## EXPERIMENT 38.

FOOD	gm.	DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Ether and alcohol extracted casein (1) ...	18.	1	13.2	12.9
Protein-free milk heated with alcohol 1 day but no extract removed .....	29.5	12	17.	15.
Starch .....	23.6	17	16.	16.1
Lard .....	25.	24	17.6	17.7
		45	12.	13.1

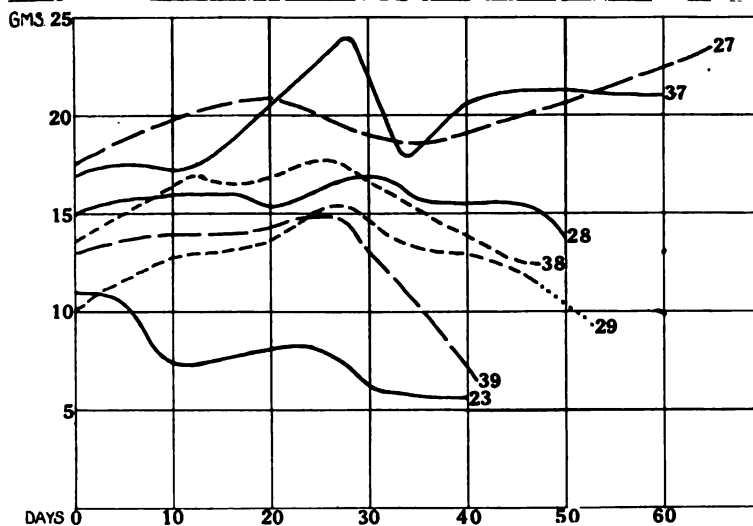


FIG. 2.

Experiment 29 clearly shows that some substance is lacking in synthetic foods that have been extracted with organic solvents. Lecithin (Experiment 23) does not supply the lack nor does cephalin (Experiment 28), though it seems better able to maintain mice than lecithin. In other experiments with cephalin none of the mice grew, most of them slowly declined, and some died after about sixty days' feeding. The third fraction (Experiment 39), made up largely of cholesterol and fats, contained no material necessary for the animals' maintenance or growth.

It is very evident that the warm alcoholic extract (Experiment 27) causes the mouse to grow about as well as the whole dried egg yolk (Experiment 26). The portion of this warm alcoholic extract that precipitates on cooling (Experiment 36) as well as the portion remaining dissolved (Experiment 37) seems to supply the food with the missing substance. This suggests an incomplete separation of the desired substance from the hot alcohol on cooling. That this substance is probably unstable and decidedly affected by heat is indicated in Experiment 38. In all cases except this last (Experiment 38) several series were run and the results agreed with the ones given.

In order to check the previous results as well as to get a clearer idea as to the solubility of the product sought, the following experiments were carried out.

## EXPERIMENT 41.

FOOD		DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Pure casein (2).....	18.	1	17.	11.7
Extracted protein-free milk (v).....	29.5	8	19.	13.6
Starch.....	23.5	15	20.	13.7
Lard.....	25.	22	21.	14.
Ether-soluble portion (h).....	5.	29	20.	12.2
		36	19.5	13.
		41	17.7	11.6
			Unhealthy looking.	

## Lipins in Nutrition

## EXPERIMENT 42.

FOOD		DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Pure casein (2).....	18.	1	13.2	12.2
Extracted protein-free milk (v).....	29.5	8	15.6	14.3
Starch.....	23.5	15	15.7	14.4
Lard.....	25.	22	16.3	15.7
Cold alcohol extract (g).....	5.	29	16.4	(13.3)
		36	19.1	18.2
		41	19.	18.1
Looking excellent.				

## EXPERIMENT 44.

FOOD		DAYS	WEIGHT	
			Male	Female
	gm.		gm.	gm.
Pure casein (2).....	18.	1	13.3	13.5
Extracted protein-free milk (v).....	29.5	8	13.	13.1
Starch.....	23.5	15	14.5	14.
Lard.....	25.	22	12.1	12.
Warm alcohol extract (i).....	5.	29	9.9	9.6
		36	10.5	9.7
		41	10.7	10.2
In poor condition.				

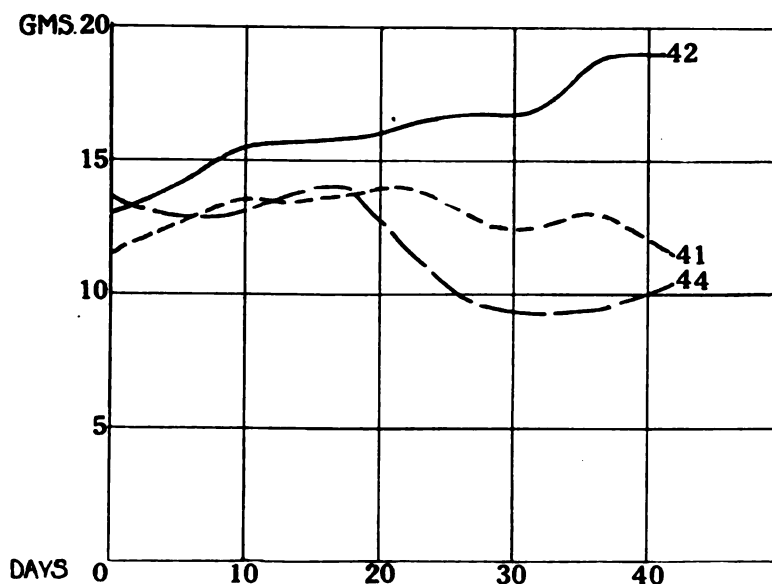


FIG. 3.

There is little doubt that the ether solution (Experiment 41) containing the lecithin, cephalin, and small amounts of other phosphatids, cholesterol, and ordinary fats, is not essential. That the cold alcohol extract, containing lecithin and small amounts of other phosphatids, fats, cholesterol, cerebroside, and some unknown substance or group of substances, is a necessary part of the egg both for maintenance and growth is indicated in Experiment 42. Previous experiments show that neither the lecithin, cephalin, cerebroside, cholesterol, nor ordinary fats are the desired compound. It seems that this necessary compound is an unknown one, which is insoluble in ether, and either completely soluble in cold alcohol or destroyed by short heating with alcohol; because the hot alcohol extract (Experiment 44) (containing cerebroside and small amounts of phosphatids and other substances), following the cold alcohol extract, did not keep mice in normal condition. Many of the properties of this substance suggest vitamin E.

Further work is being done to characterize this unknown substance and, if possible, to identify it.

## SUMMARY.

(The data presented strongly indicate that) <sup>2</sup>lecithin, cephalin, cerebrosides, cholesterol, and fats, are dispensable parts of a food for mice, but ~~that~~ a substance is present in egg yolk, insoluble in ether, soluble in cold alcohol, and probably easily destroyed by heat, that needs to be added to a synthetic food containing casein, starch, lactose, lard, and the salts of milk, to make it a complete food.

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## NOTE ON THE USE OF COLLOIDAL IRON IN THE DETERMINATION OF LACTOSE IN MILK.

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The method described below has the advantage of being within the reach of the ordinary laboratory student; it requires comparatively little time and gives very accurate results.

In clarifying the milk, a 10 per cent solution of colloidal iron (dialyzed ferric hydroxide) is used. By adding the proper amount of colloidal iron, all the proteins of the milk are completely precipitated and can be rapidly filtered off leaving a perfectly clear colorless filtrate.

The method is as follows: To a 10 gram sample of milk, which has been diluted to about 25 cc., about 3 cc. of a 10 per cent solution of colloidal iron are added. The amount of colloidal iron necessary depends upon the composition of the milk and can be accurately determined by adding the last portion drop by drop, and agitating after each addition.

If the precipitation is complete, a clear supernatant liquid separates out from the flocculent precipitate; if too little has been added, the supernatant liquid will appear milky; if too much, it will have a reddish tinge.

The sample is next filtered into a 100 cc. volumetric flask, and the precipitate thoroughly washed with distilled water until the filtrate and washings aggregate about 100 cc. The flask is then filled to the mark and the percentage of lactose determined by Benedict's quantitative method.<sup>1</sup> About 16 cc. of the diluted sample will be required to reduce completely 25 cc. of Benedict's quantitative solution.

<sup>1</sup> S. R. Benedict: *Jour. Am. Med. Assn.*, lvii, p. 1193, 1911. P. B. Hawk: *Practical Physiological Chemistry*, 4th edition, Philadelphia, 1912, p. 386.

A very convenient method of analysis is given by Cole,<sup>2</sup> in which a 4 ounce flask is used instead of an evaporating dish. The wide mouthed Jena 150 cc. flat bottomed flasks are very convenient for the determination. The flask is fitted into the 2.5 inch ring of a retort stand, and the height above the Bunsen burner so arranged that the contents of the flask will be kept briskly boiling with a small flame. Two flasks can be run simultaneously from the same stand.

Three to four grams of anhydrous sodium carbonate are dissolved, by means of heat, in 25 cc. of twice diluted<sup>3</sup> Benedict's solution, to which a little powdered pumice has been added. About 14 cc. of the sugar solution are then rapidly added from a burette. Boiling is continued for at least one-half minute before the addition of more lactose solution.

When reduction is complete the supernatant liquid will have a slight yellowish tinge to which the blue color very slowly returns. If the end-point has been underestimated, it will have a blue or greenish tinge that rapidly becomes bluer. With a little practice, and by adding the last portion a drop at a time, and boiling one-half minute after each addition, the end-point can be determined to within one drop.

Twenty-five cc. of Benedict's quantitative solution are completely reduced by 0.0676 of a gram of anhydrous lactose. Since the milk has been ten-fold diluted, 0.0676 divided by the number of cc. of diluted lactose solution used, multiplied by ten, will give the percentage of lactose in the milk. If 16.1 cc. of lactose solution were required, then  $16.1:0.0676::X:10 = 4.20$  per cent.

That very accurate results can be obtained by using this method may be seen from the following tables. Table I shows the comparison between duplicate samples of different milks. Table II shows the effect of the addition of the small quantities of lactose to the milk before analysis.

<sup>2</sup> S. W. Cole: *Practical Physiological Chemistry*, St. Louis, 1914, p. 53.

<sup>3</sup> I prefer to dilute 25 cc. of Benedict's solution to at least 50 cc., for I obtain a more accurate end-point with dilute than with concentrated solutions.

TABLE I.

*Comparison between duplicate samples of different milks.*

SAMPLE NO.	ANHYDROUS LACTOSE IN MILK 1	LACTOSE IN MILK 2	LACTOSE IN MILK 3
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	4.08	4.19	4.23
2	4.11	4.20	4.20
3	4.10	4.20	4.22

TABLE II.

*Effect of the addition of lactose to 10 cc. samples of milk.*

SAMPLE NO.	SAMPLE, TEN TIMES DILUTED, REQUIRED TO REDUCE 25 CC. OF BENEDICT'S QUANTITATIVE SOLUTION	ANHYDROUS LACTOSE IN SAMPLE	ANHYDROUS LACTOSE ADDED	ADDED LACTOSE RECOVERED
	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Milk 4				
1	16.7	0.405	None	
2	14.9	0.454	0.050	98
3	15.0	0.451	0.050	92
Milk 5				
1	16.1	0.420	None	
2	14.4	0.469	0.050	98
Milk 6				
1	16.4	0.413	None	
2	7.5	0.902	0.505	97
3	10.2	0.664	0.2525	99
4	10.1	0.670	0.2525	100.8



## SPECTROSCOPIC INVESTIGATION OF THE REDUCTION OF HEMOGLOBIN BY TISSUE REDUCTASE.<sup>1</sup>

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### I. INTRODUCTION.

In our communication, "Studies on the reductase of liver and kidney. Part I," we showed<sup>2</sup> that ox liver juice two days old was able to reduce methemoglobin to the well known two-banded oxyhemoglobin, within ten minutes at 40°. This methemoglobin was prepared from "hemoglobin scales, soluble. Merck." This observation we have confirmed and extended. It has been found that fresh cat liver juice will reduce methemoglobin not only to the state of oxyhemoglobin, but to that of the completely reduced pigment (Hb). The juice used was press juice made by the technique employed in our previous work, and the methemoglobin was procured by exposing cat defibrinated blood (diluted 1 in 25) to the air in an ice box for several days. A mixture of 10 cc. of juice with 25 cc. of the methemoglobin placed in the thermostat at 40° showed within three minutes the absence of the band in the red region and the appearance of the two well marked bands of oxyhemoglobin; while in six minutes more these two bands had gone, to be replaced by the single band of reduced hemoglobin. The changes visible to the naked eye corresponded with the spectroscopic appearances; the mixture originally of a rusty

<sup>1</sup> Part of the expenses of this research were met from the residue of a grant from the Government Grant Committee of the Royal Society previously acknowledged.

<sup>2</sup> D. Fraser Harris and H. J. M. Creighton: *Proc. Roy. Soc., Series B*, lxxxv, p. 487, 1912.

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brown passed through a pink to become the purple colour so characteristic of solutions of fully reduced blood. We found that this fully reduced pigment could be oxidised to two-banded oxyhemoglobin on shaking with air, and that, when placed once more in the thermostat, it became again reduced to the one-banded condition.

The work about to be described we undertook because we thought it well to investigate the behavior of reductase in the presence of oxyhemoglobin, which is the natural source of the oxygen with which the enzyme deals in the living tissues. Such chemical substances as we had previously used to demonstrate reduction by tissue juices must be all, more or less, poisonous to a tissue enzyme, so that by using oxyhemoglobin we have the reductase in the presence of what might be called a natural substrate. Further, by using oxyhemoglobin to be reduced by tissue juices, we thought that certain data might be obtained which would shed light directly on the general problem of tissue respiration, a process which is being more and more thought of as enzymic in its essential features. We saw no reason to doubt that fresh tissue juices could at least bring about such reductions as do the intact tissues themselves. Oxyhemoglobin is reduced, though not completely, in one capillary transit of the circulating blood, so we thought it well to try to ascertain the time relations of the complete reduction of oxyhemoglobin to hemoglobin, the temperature coefficient of the change which we ascribe to a tissue enzyme, the rate of decay in activity of this enzyme, and any other data likely to be of service in a conception of the process of internal respiration.

### II. TECHNIQUE.

The late Professor Yeo in 1885 had employed<sup>3</sup> a spectroscopic method to demonstrate the reduction of mammalian oxyhemoglobin by the activity of the beating frog heart. Certain observers<sup>4</sup> had immersed pieces of tissue in solutions of oxyhemoglobin, and after varying intervals of time had examined the solutions spectroscopically.

<sup>3</sup> G. F. Yeo: *Jour. Physiol.*, vi, p. 93, 1885.

<sup>4</sup> J. G. Mackendrick: *Proc. Roy. Soc. Edinburgh*, xxii, p. 201, 1897-98.

In the replacing of the two bands of oxyhemoglobin by the one band of fully reduced hemoglobin, we have an optical end-point which is not affected by any personal factor. But the disappearance of the two bands is from a very early period accompanied by the appearance of a fainter band between them, *i.e.*, in the intralinear green, so that at a certain stage of reduction we have the single band of reduced hemoglobin flanked by the remains of the two bands of the oxyhemoglobin. We have not considered the pigment fully reduced until these margins of the single band have completely disappeared, so that in this way we contrived to have an end-point of considerable definiteness. We used the small direct vision spectroscop. It was early evident that owing to the opacity of the mixture of tissue juice and diluted blood in even a narrow test-tube, the spectrum of the oxyhemoglobin could not be seen sufficiently distinctly to be certain of the end-point. We found that small vessels made by drawing out short lengths of glass tubing (6 mm. diameter) to a conical end answered our purpose very well; for if in doubt as to the persistence of the two bands, when we directed the spectroscop across the tube itself, it was only necessary to look through the conical part of the tube where the mixture was sufficiently transparent to observe a distinct spectrum. Another objection to the use of even a narrow test-tube as an observation tube was, that if we filtered off some of the mixture of juice and diluted blood in order to obtain a clear liquid, any reduced hemoglobin was certain to be rapidly reoxidised to the two-banded condition. This source of error was felt to be so serious that we early abandoned the method of filtering, and decided that the spectrum must be examined in a mixture of juice and diluted blood to which oxygen could not gain access.

All animals used were decapitated during chloroform anesthesia.

The dilution of blood found most suitable for spectroscopic purposes in a semi-opaque mixture was 10 cc. of defibrinated blood diluted with water to 250 cc. This dilution, viewed through the conical observing tube, showed distinctly the two bands of oxyhemoglobin separated by some intralinear green light in such a way that the subsequent filling up of this coloured region by the one band of the reduced hemoglobin was distinctly noticeable. Complete reduction was considered to have taken place at the first moment when the two bands had vanished, and when they



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were no longer distinguishable as ribbed margins to the single band.

When only the liver was required for juice, it was perfused through the portal vein with tap water at 40° until bloodless. When muscles, heart, pancreas, other viscera, or cortex cerebri were needed, the perfusion cannula was tied into the origin of the aorta. The juice from the tissues, whether crushed in the juice press or macerated with sand in a mortar, was received and kept under toluene. A mixture of 10 cc. of juice with 25 cc. of diluted blood (1 in 25) was employed in all cases unless otherwise stated. This mixture in a conical tube was always covered with a layer of toluene to a depth of 1 cm.

### III. EXPERIMENTAL.

#### *A. Reduction with liver juice.*

(a) *Cat liver juice and oxyhemoglobin.* A mixture of freshly prepared cat liver juice and its diluted blood was placed in the thermostat at 40° at 4.15 p.m., and was examined with the spectroscope every minute. At 4.28 it was found to be one-banded. The boiled control tube remained two-banded at the end of this day's series of observations, three hours later. On vigorously shaking the reduced mixture with air, it at once became distinctly two-banded. A second mixture of liver juice and blood in the usual proportions was placed in the thermostat at 40° at 4.33 p.m. At 4.44, *i.e.*, in eleven minutes, it was found to be one-banded. It was capable of reoxidation, and on being returned to the bath was found to undergo reduction in the same time as the first, eleven minutes. Other observations on the same day with juice not more than four hours old gave similar results.

In order to eliminate every source of error, we thought it well to determine whether dextrose could possibly be the agent active in reducing the oxyhemoglobin, as dextrose is a constant constituent of the liver of all but fully starved animals. Accordingly we mixed 2 cc. of a 2 per cent solution of dextrose with 4 cc. of the diluted blood used in these experiments, and kept the tube at 40° for 105 minutes, at the end of which time the solution was still two-banded. Since fresh liver juice can reduce oxyhemoglobin at 40° certainly within fifteen minutes, the reduction must be due to something in the juice other than dextrose.

Exactly similar observations with bile convinced us that none of the reduction of oxyhemoglobin by liver juice was due to any residual bile that may have been in the liver.

(b) *Rabbit liver juice.* Observations with mixtures of rabbit liver juice and rabbit blood yielded results similar to those just described.

(c) *Pigeon liver juice.* Our experiments with this juice showed us that it contained the most active reductase we have yet met with. A mixture of fresh pigeon liver juice and pigeon diluted blood in the usual proportions showed complete reduction so rapidly that there was no time to place the mixture in the thermostat at 40°; that is, at room temperature (17°) the reduction was effected at once. A boiled control showed no change at the end of several hours.

This great energy of reduction as displayed towards oxyhemoglobin we found to be equally well exhibited towards soluble Prussian blue. 1 cc. of the liver juice caused 2 cc. of a 0.5 per cent solution of soluble Prussian blue to fade instantly at room temperature. More of the Prussian blue solution was added until 8 cc. had been poured in; the blue colour faded to the natural fawn liver colour after each addition of the pigment. The boiled control showed no change in colour after similar additions of soluble Prussian blue solution. The entirely bleached mixture was restored to a blue colour on the addition of hydrogen peroxide.

(d) *Frog liver juice.* The juice of frog liver was tested against frog blood both at room temperature (20°) and at 40°. At the former, the oxyhemoglobin was completely reduced within thirty-seven minutes; at 40° the reduction was complete in almost the same time (thirty-five minutes). A large amount of very dark pigment in the liver rendered the spectroscopic observations difficult.

(e) *Fish liver juice.* The non-perfused liver of a mackerel, caught about twenty-four hours previously, was crushed in the juice press: a homogeneous, viscous, livid juice was obtained. This viscous juice was mixed with two-thirds of its volume of water in order to have a juice of suitable fluidity. The blood of the liver was seen to be already reduced in this juice. Of this fluid liver juice, 2 cc. were mixed with 4 cc. of the fish blood (dilution 1 in 25); reduction took place at once at air temperature (20°).

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In a second experiment 2 cc. of the liver juice were mixed with 8 cc. of the blood solution; this mixture was introduced into the observation tubes, one of which was placed in the bath at 40° and the other allowed to remain at room temperature. The oxyhemoglobin in the tube at 40° was found to have become one-banded at the end of one minute, while that in the tube at air temperature required two minutes for complete reduction.

In another experiment 1 cc. of the liver juice was mixed with 8 cc. of the blood solution. Observation tubes containing portions of this mixture were placed in the bath at 40° and in a bath at room temperature. At the end of two and a half minutes reduction had taken place at 40°, while seven and a half minutes were required to effect this at 20°. Observations made with fish blood solution and liver juice which had been boiled for a minute showed no evidence of reduction at the end of several hours.

The reducing power of the reductase in this liver juice was corroborated by our finding that 1 cc. of it completely decolourized at 20° 10 cc. of a 0.05 per cent solution of soluble Prussian blue.

(f) *Experiments on crossed reductions.* By crossed reduction we mean the action of reductase from any animal A on the oxyhemoglobin of any other animal B. It was highly probable that this would occur, seeing that frog heart, for instance, can reduce rabbit or ox dilute defibrinated blood in physiological perfusion experiments. The late Professor Yeo had shown<sup>5</sup> by the use of the spectroscope that the frog heart could reduce mammalian oxyhemoglobin to the one-banded condition.

With cat liver juice at 40°, it was found that this substance reduced pigeon blood in half the time (three and five-tenths minutes) in which it reduced cat blood (six and five-tenths minutes). Cat liver juice reduced frog blood in six minutes at 40°, or in the same time in which it reduced cat blood. Similarly, pigeon liver juice reduced cat blood in about the same time that it reduced pigeon blood. In experiments on pigeon muscle juice, we found that it reduced pigeon blood more rapidly than it did cat blood.

<sup>5</sup> Yeo: *loc. cit.*

*B. Reduction with muscle juice.*

(a) *Cat muscle juice.* Contrary to our expectations it was not difficult to obtain a press juice from cat muscles. A highly colloidal juice, which coagulated on boiling, was received under toluene. This juice mixed in the routine proportions with diluted blood and placed in the thermostat at 40° showed little reduction at the end of half an hour. A boiled control showed none whatever, but the oxyhemoglobin in this case underwent a change which we can only call fading; for its colour became a pale brown and its two bands became fainter and fainter until they were just discernible. Neither at room temperature nor at 40° did cat muscle juice (two hours post mortem) fully reduce cat blood. The fading of the oxyhemoglobin was, we think, largely due to adsorption, whereby nearly all of it was removed from solution. One factor in the failure to reduce the oxyhemoglobin may have been that the juice was prepared from muscles two hours after death.

This muscle juice tested with soluble Prussian blue effected only a very slight reduction of that pigment, for the mixture of the muscle juice and Prussian blue did not become colourless or even gray, but only a pale green. The boiled control, however, remained blue, although it had adsorbed some of the colour.

(b) *Pigeon muscle juice.* The non-perfused pectoral muscles of a pigeon, before the animal heat had left them, were disintegrated, ground up with sand and a little water and filtered through cheese-cloth. Of this filtrate 2 cc. viewed in the conical observation tube at room temperature showed that the residual blood of the muscle ~~was~~ already reduced. Two cc. of the muscle juice were mixed with 2 cc. of pigeon blood at room temperature, and in two minutes the oxyhemoglobin was found completely reduced. On the other hand, the boiled control remained two-banded for many hours. As estimated by its action on soluble Prussian blue, the muscle juice was an active reducer, but not so active as the liver juice of either the cat or the pigeon.

(c) *Cardiac muscle juice.* The still warm hearts of four kittens were crushed in the juice press and ground up with sand and a little water. The mixture was filtered through cheese-cloth. The usual mixture of juice and diluted blood did not at any time

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show the disappearance of the two bands of oxyhemoglobin; indeed, there was little difference between the active tubes and the boiled controls. Quite different was our experience with the heart juice of a small rabbit. The entire heart while still warm was ground up with sand and a little water, and some of the filtrate through cheese-cloth was at once mixed with some of the animal's defibrinated diluted blood (1 in 25). This was reduced in less than three minutes at room temperature, while a boiled control remained unchanged at the end of two hours. A frog heart crushed and mixed with diluted frog blood at room temperature had not reduced the oxyhemoglobin at the end of thirty minutes.

### *C. Reduction with the juices of other organs.*

(a) *Kidney.* The eight non-perfused kidneys of four decapitated kittens were crushed and the juice was prepared in the usual way. The blood was defibrinated and diluted, though not quite to such an extent as was the blood employed in the preceding experiments. A mixture of juice and blood made in the usual proportions showed complete reduction in fifty-seven minutes at 40°. On shaking the tube with air, the reduced pigment was reoxidised to two bands as distinct as they had been at first, but on returning the mixture to the bath its reduction was effected only with very great slowness.

(b) *Stomach.* The tissues of the wall of the stomach of a cat were disintegrated while still warm and ground up with sand and a little glycerine. Some of the resulting juice was mixed with diluted cat blood and placed in the thermostat at 40°. The juice slowly separated from the blood solution, making it impossible to maintain a homogeneous mixture; but where the juice and blood came in contact, reduction had clearly taken place in less than half an hour.

(c) *Pancreas.* Only one observation was made with disintegrated cat pancreas and blood, and it was negative.

(d) *Cortex cerebri.* Some of the fresh cortex cerebri was ground up with a little glycerine. A mixture of 1.5 cc. of the juice and 10 cc. of diluted cat blood was placed in the thermostat at 40°. In forty-seven minutes the oxyhemoglobin was fully reduced, whereas

that in the boiled control was still two-banded at the end of several hours.

The reducing power of the cerebral tissue was confirmed in an experiment with soluble Prussian blue. A mixture of 1.5 cc. of the juice with 10 cc. of 0.1 per cent solution of Prussian blue was reduced within a minute to the gray condition, more blue was added, when the mixture again became gray; a third time blue was added with the same result. A mixture containing 1.5 cc. of boiled juice and 10 cc. of the Prussian blue solution remained blue-green.

*D. Effect of temperature on the reducing power of reductase, and the rate of decay of its activity.*

In the following experiments the reductase contained in cat liver juice was employed.

The reducing power of reductase, at different temperatures, was measured by determining the time required for the reduction of two-banded hemoglobin to the one-banded condition. This procedure was also employed to determine the variation in the reducing power of reductase with time. The defibrinated cat blood used in these measurements was kept in an ice box, where it was found to have undergone no apparent deterioration at the end of eight days. The blood was always oxygenated by shaking with air, before being used for the preparation of the hemoglobin solutions. In the following experiments 10 cc. of the liver juice were mixed with 25 cc. of oxyhemoglobin solution, prepared by diluting 25 cc. of defibrinated cat blood to 250 cc. with water. This mixture was then divided into several parts, which were introduced into the observing tubes, and these were then quickly immersed in different thermostats. The temperatures of the thermostats were kept constant to  $\pm 0.2^\circ$ .

Table I gives the time required at different temperatures for the reduction of a solution of oxyhemoglobin, prepared from fresh defibrinated cat blood, by fresh cat liver juice (half an hour post mortem).

In Table I column 6 gives the temperature coefficient of the process of reduction for intervals of  $10^\circ$ . These values have been obtained by dividing the average time required for complete

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reduction at any one temperature by that required at a temperature  $10^{\circ}$  higher. Column 7 gives the reducing power of the reductase of cat liver juice in units obtained by multiplying by 100 the reciprocal of the time required for reduction at a particular temperature. The change in the reducing power of the reductase of cat liver juice with temperature is shown graphically in Figure 1.

In addition to the foregoing, measurements of the reduction of oxyhemoglobin by reductase were carried out at  $60^{\circ}$  and  $70^{\circ}$ . At the former temperature one and three-quarters minutes were required to effect reduction, and at the latter, one and one-quarter minutes. These measurements above  $60^{\circ}$  are probably of little

TABLE I.

TEMPERATURE	TIME REQUIRED FOR COMPLETE REDUCTION OF HbO <sub>2</sub>				TEMPERATURE COEFFICIENT $\frac{t_{\theta^{\circ}}}{t_{\theta^{\circ}} + 10^{\circ}}$	REDUCING POWER OF REDUCTASE $\frac{100}{t_{\theta^{\circ}}}$
	Experiment I		Experiment II	Average time		
	a	b				
	$^{\circ}\text{C.}$	min.	min.	min.		
0	98	98		98	2.65	1.02
10	36	38		37	1.91	2.70
20	22	21	15	19.3	2.07	5.17
30	10	10	8	9.3	2.00	10.72
40	5	5	4	4.7	1.86	23.09
50	2.5	2.5	2.5	2.5		40.00
55	1.75	1.75		1.75		57.14

value owing to the decomposition of the oxyhemoglobin; for, as one of us has shown,<sup>6</sup> at temperatures above  $60^{\circ}$  the two bands of oxyhemoglobin rapidly become hazy.<sup>7</sup>

Measurements were made from day to day of the reducing power of the reductase of the liver juice on an oxyhemoglobin solution made from defibrinated cat blood of the same age as the juice. These determinations were carried out at different temperatures, in order to ascertain whether the ratio of the times

<sup>6</sup> D. Fraser Harris: *Proc. Roy. Soc. Edinburgh*, xxii, p. 192, 1897-98.

<sup>7</sup> The temperature coefficient from  $0^{\circ}$  to  $55^{\circ}$  (Table I) varies at both extremes of this range in a manner similar to that observed by Van Slyke and Cullen in the case of urease (this *Journal*, xix, p. 174, 1914). The similarity in the behavior of the two enzymes indicates a probable likeness in their mode of action.

required for reduction at any two temperatures varied with the age of the reductase. The results of the observations, which are given in Table II, show a marked decrease in the activity of the enzyme from day to day. The times given in this table for reduction represent the means of two experiments which seldom differed by more than a few seconds and which, in most cases, were identical.

The change with temperature in the reducing power of the reductase of cat liver juice of different ages is shown graphically in Figure 1. It will be seen from the shape of the curves that the ratio of the times required for reduction at any two temperatures undergoes little or no variation with the age of the reductase.

TABLE II.

TEMPERATURE	24 HR. OLD JUICE		44 HR. OLD JUICE		92 HR. OLD JUICE		188 HR. OLD JUICE	
	Time required for the reduction of HbO <sub>2</sub>	Reducing power of the juice $\frac{100}{t_g^\circ}$	Time required for the reduction of HbO <sub>2</sub>	Reducing power of the juice $\frac{100}{t_g^\circ}$	Time required for the reduction of HbO <sub>2</sub>	Reducing power of the juice $\frac{100}{t_g^\circ}$	Time required for the reduction of HbC <sub>2</sub>	Reducing power of the juice $\frac{100}{t_g^\circ}$
°C.	min.		min.		min.		min.	
10	41	2.44	55	1.90	124	0.81		
20	19.3	5.18	28	3.57	58	1.72		
30	9	11.11	12	8.33	30	3.33	230	0.43
40	5.4	18.52	6.5	15.38	15	6.66	94	1.06
50	3.3	30.3	4	25.0	8.5	11.76	39	2.56
55	2.3	43.5	3	33.3	5	20.0	27	3.70

Figure 2 shows the change of the reducing power of the reductase of cat liver juice with age, for several temperatures. In this figure two sets of curves are given: the reducing power of the reductase expressed in the units given in the tables; and the logarithms of these units are plotted on the axis of ordinates against the age of the enzyme on the axis of abscissae. The curves found by plotting the logarithms of the reducing powers against the age of the reductase are straight lines over the whole period of time.

The numbers in the foregoing tables show, as is to be expected, that the reductase of cat liver exhibits as an essential characteristic a marked sensitivity towards changes in temperature; and, analogous with the majority of chemical reactions, the velocity of the reduction of oxyhemoglobin to the one-banded condition



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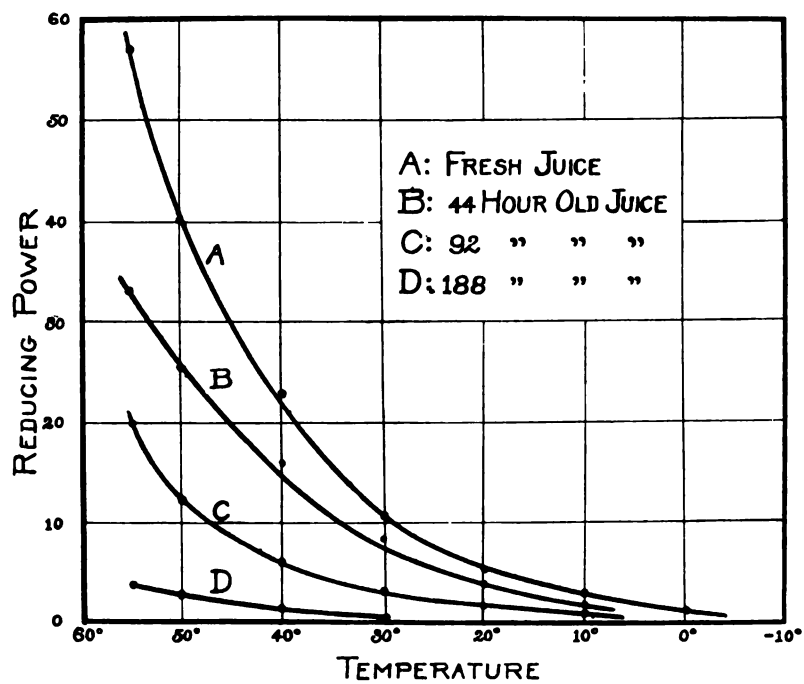


FIG. 1.

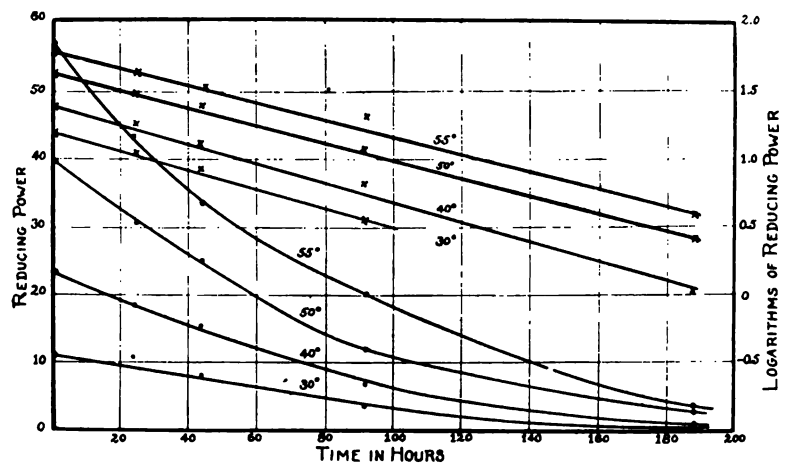


FIG. 2.

by the enzyme increases with temperature. Between  $10^{\circ}$  and  $40^{\circ}$  the velocity of reduction is approximately doubled for every  $10^{\circ}$  rise in temperature, the temperature coefficient being about 2. Above  $40^{\circ}$  the temperature coefficient and the consequent acceleration of the velocity of reduction decrease rapidly with the rise in temperature. Between  $50^{\circ}$  and  $60^{\circ}$  the temperature coefficient has been found to be 1.43. Although, in general, the temperature coefficients of chemical reactions decrease slightly with increase in temperature, the decrease in the values obtained for the reduction of oxyhemoglobin by reductase, at temperatures above  $40^{\circ}$ , is much greater than would be the case in an ordinary chemical reaction. It is probable, therefore, that above  $40^{\circ}$  some new influence makes itself felt. It has been shown by one of us<sup>8</sup> that the optimal temperature of reductase lies between  $42^{\circ}$  and  $46^{\circ}$ . In view of this, it is likely that, at temperatures between  $40^{\circ}$  and  $60^{\circ}$ , the acceleration of the velocity of reduction due to increase in temperature is to a certain extent counteracted by a partial inhibition or destruction of the enzyme, the result being a relatively large decrease in the value of the temperature coefficient. Below  $10^{\circ}$  the velocity of reduction of oxyhemoglobin diminishes very rapidly with decrease in temperature, and, indeed, below  $0^{\circ}$  it is so extremely slow that the inhibition of the enzyme may be regarded as almost complete. That cold does not permanently inhibit the reductase is shown by the fact that when liver juice, which has been kept at  $0^{\circ}$  for several hours, is mixed with a solution of oxyhemoglobin at  $40^{\circ}$ , reduction proceeds at the usual velocity. This substantiates similar observations previously made<sup>9</sup> with liver juice and soluble Prussian blue.

The decrease in the reducing power of cat liver juice at any temperature, with increase in the age of the juice, is doubtless due to a fall in the activity of the enzyme, a phenomenon which is common to most enzymes in solution. Since the same preparation of liver juice, which was kept at room temperature, was used in all the foregoing temperature measurements, the fall in the activity of the reductase should occasion the same percentage decrease in the reducing power of the juice, independent of the

<sup>8</sup> Harris: *Biochem. Jour.*, v, p. 158, 1912.

<sup>9</sup> Harris and Creighton: *Proc. Roy. Soc., Series B*, lxxxv, p. 491, 1912.

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temperature at which the reduction was carried out. The shape of the curves in Figure 2 confirms this. The reductions carried out at 30°, 40°, 50°, and 55° show that, at the end of eight days, the reducing power of the juice had decreased to 5–6 per cent of its original value. This, of course, indicates a corresponding decay in the activity of the reductase.

The character of the curves in Figure 2, portraying the decrease in the reducing power of reductase with the age of the juice, shows that the decay of the activity of the enzyme follows the logarithmic law.

The decay in the activity of reductase is of interest. Shaklee<sup>10</sup> has found that the decay in the activity of pepsin at 37° is given by the equation,

$$\frac{x}{t(a-x)} = k,$$

where  $x$  is the percentage quantity of pepsin rendered inactive,  $a$  the original quantity,  $t$  the time, and  $k$  a constant (= about 0.5). After maintaining pepsin at this temperature for twelve days, it was found to contain only 14 per cent of the active enzyme. This formula has been applied without success (compare Table III) to our reduction measurements. In applying this formula the reducing power of the fresh juice has been taken as 100 per cent, and it has been assumed that the reducing power of the reductase is proportional to the amount of active enzyme present.

In order to ascertain whether the decay in the activity of reductase with age follows the unimolecular law, the results given in the foregoing tables have been used in the equation,

$$\frac{1}{0.4343 t} \log \frac{a}{a-x} = k,$$

to calculate the value of the velocity constant,  $k$ . Since the activity of the enzyme is proportional to its reducing power,  $a$ , the initial activity, may be represented by 100 per cent, and  $x$ , the decrease in activity at the end of time,  $t$ , may be represented by the percentage decrease in reducing power at the end of this time. The values obtained for  $k$  with the unimolecular and Shaklee's equations are given in Table III.

<sup>10</sup> A. O. Shaklee: *Centralbl. f. Physiol.*, xxiii, p. 4, 1909–10.

Although the values for the unimolecular constant given in Table III show considerable differences, they are sufficiently constant to indicate that the decay of activity of reductase undoubtedly follows the unimolecular law. When the inaccuracies of the method employed for measuring the reducing power of reductase, and the uncertainties of the initial and end-points of the

TABLE III.

TIME	$a - x$	$x$	$k = \frac{x}{t(a - x)}$	$k = \frac{1}{0.4343t} \log \frac{a}{a - x}$
hrs.	per cent	per cent		
Temperature 55°				
0	100	0		
24	76.2	23.8	0.0130	0.0113
44	58.3	41.7	0.0162	0.0141
92	35.0	65.0	0.0202	0.0097
188	6.5	93.5	0.0765	0.0146
				Mean: 0.0132
Temperature 50°				
0	100	0		
24	75.7	24.3	0.0133	0.0116
44	62.5	37.5	0.0136	0.0107
92	29.5	70.5	0.0259	0.0133
188	6.5	93.5	0.0765	0.0146
				Mean: 0.0134
Temperature 40°				
0	100	0		
24	80.0	20.0	0.0104	0.0093
44	66.6	33.4	0.0114	0.0093
92	29.0	71.0	0.0266	0.0135
188	4.8	95.2	0.1055	0.0162
				Mean: 0.0121

process are considered, the divergencies in the values of the velocity constant are not surprising. On the other hand, the continual increase of  $k$  with time, when calculated by means of Shaklee's formula, makes it evident that this equation cannot be employed to determine the decay in the activity of reductase. As is to be expected, the same value is obtained for the unimolecular velocity constant at different temperatures; for  $x$  represents the decrease

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in the reducing power of the liver juice at room temperature, the temperature at which the juice was kept. The variations in the room temperature would partly account for changes in the value of  $k$ . Since it is evident that the decay in the activity of reductase follows the unimolecular law, and that the activity of the enzyme is proportional to its reducing power, the percentage of active reductase present in a juice of any age may be calculated by means of the unimolecular equation.

### IV. DISCUSSION ON THE PHYSIOLOGICAL BEARING OF THE EXPERIMENTS.

We desire, in the first place, to point out the exacting nature of the chemical task set before tissue reductase in our experiments. In no case did we report full reduction until the two bands of oxyhemoglobin had given place to the one band of the reduced pigment. In other words, we forced the reductase to accomplish *in vitro* a degree of reduction of the blood pigment, which it is never called upon to do *in vivo* within one circuit of the blood through any capillary district. The blood leaving a capillary district is stated to possess about two-thirds of the oxygen it had on entering it; that is, it is far from being completely reduced. Not until it has made several circuits is the oxyhemoglobin all reduced and the tissues said to be asphyxiated. The dissociation of the oxygen from the hemoglobin is the event of physiological importance in the depths of the tissues.

Recently much work has been done on the problem of the physicochemical mechanism of the liberation of the oxygen from oxyhemoglobin. Barcroft<sup>11</sup> has investigated the influence of the presence of carbon dioxide in the blood as a factor in the dissociation of the oxygen from oxyhemoglobin. In cold-blooded animals he holds that it is a most important factor; but surely in all kinds of animals the oxygen avidity or reducing power of the tissues is the chief factor. Starling, quoting Barcroft, writes: "In cold-blooded animals the dissociation of oxyhemoglobin with the setting free of oxygen must be largely conditioned by the rise of carbon dioxide tension in the tissues, since at the normal temperature of these animals the evolution of oxygen from

<sup>11</sup> J. Barcroft and W. O. R. King: *Jour. Physiol.*, xxxix, p. 377, 1909-10.

hemoglobin is extremely slow." We submit that the part played by reductase in this tissue respiration is the main factor. At room temperature, cold-blooded animal tissue juices possess reducing power. No doubt carbon dioxide is being evolved in small amounts even in disintegrated tissues, but the reduction of oxyhemoglobin must be regarded as due to the same constituent of tissue juices that reduces soluble Prussian blue or methylene blue; that constituent is not carbon dioxide, and we have shown that it behaves in more than one particular as does an enzyme. Further, the active reducer in tissues is not appreciably soluble in water; carbon dioxide is eminently so. Tissue reduction is the more rapid the higher the temperature, within the limits of enzymic action; but the higher the temperature the more rapid the evolution of carbon dioxide from tissue juices and, consequently, the less there is present to effect reduction of oxyhemoglobin.

Barcroft has similarly shown<sup>12</sup> that traces of acid facilitate the dissociation of oxyhemoglobin. We feel convinced that the reductions we have studied have not been due to this cause, seeing that the older the juice the less vigorously did it reduce; whereas it ought to have reduced more vigorously with age, if the active agent in it had been acid formed by tissue autolysis. Further, traces of acid in our mixtures would have tended to form methemoglobin, a pigment we have never found in any mixture of active tissue juice and hemoglobin, however long they were left in contact.

The factor of the dissociation of oxyhemoglobin with rise of temperature has not escaped us. We feel sure that it is not a potent factor in our experiments; for we have repeatedly observed that the oxyhemoglobin in contact with the boiled tissue juice in a control tube, remained unreduced at the end of many hours, when the active tube had been reduced in a few minutes.

We do not wish to minimize the action of carbon dioxide, traces of lactic or other acid, and temperature in the dissociation of oxygen from oxyhemoglobin, but we submit that there is both in cold-blooded and in warm-blooded animals an important enzymic factor in this phase of tissue respiration. We might remark that

<sup>12</sup> J. Barcroft and L. Orbeli: *Jour. Physiol.*, xli, p. 355, 1910-11.

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tissue juice reduction has been shown not to be due to alkaline salts,<sup>13</sup> or to proteins,<sup>11</sup> or to products of autolysis,<sup>15</sup> or to bacterial decomposition,<sup>16</sup> or to catalase,<sup>17</sup> as well as not due to dextrose or bile.

The differences in the behavior of the muscle juice of cat and of pigeon are noteworthy. The muscle juice of the cat, even when prepared before the heat had left it, was but a feeble reducer; whereas the juice of pigeon muscle was quite powerful. Cat muscle juice, moreover, both active and in boiled control, adsorbed much hemoglobin and so removed it from solution; the pigeon's did not. Our experiences with cat muscle are in agreement with the observations of some previous workers on the behavior of muscle in tissue respiration. Thus Sir Victor Horsley and A. B. Harris noticed<sup>18</sup> that when they injected methylene blue into living animals the voluntary muscles were very blue during activity, from which they concluded that in that condition oxidation was maximal and was predominating over reduction. Further, the late C. A. Herter in his work on the reducing power of tissues<sup>19</sup> found that liver, lung, the suprarenals, and the gray matter of the central nervous system were all better reducers of methylene blue than were muscles or connective tissue. Herter also found that the postmortem decline of reducing power in rabbit muscle was very rapid. With the general tendencies of these conclusions we agree, and can confirm the observation on the rapid decline in activity of muscle after the death of the animal. This we would associate with the physicochemical immobilization which muscle undergoes as soon as the animal heat has left it. While unquestionably reduction of oxyhemoglobin in active muscle must be a constant occurrence of physiological importance, yet it is clear that oxidative processes necessarily predominate in such a tissue as muscle, in which heat is constantly being produced and energy of movement very frequently exhibited.

<sup>13</sup> Harris: *Science Progress*, i, p. 730, 1907.

<sup>14</sup> Creighton: *Tr. Nova Scotia Inst. Sc.*, xiii, pt. 2, p. 61, 1911-12.

<sup>15</sup> Harris: *Biochem. Jour.*, v, p. 143, 1911.

<sup>16</sup> Harris: *ibid.*, v, p. 143, 1911.

<sup>17</sup> Harris and Creighton: *Proc. Roy. Soc., Series B*, lxxxv, p. 486, 1912.

<sup>18</sup> Victor Horsley and A. B. Harris: *Brit. Med. Jour.*, ii, p. 205, 1895.

<sup>19</sup> C. A. Herter: *Am. Jour. Physiol.*, xii, pp. 128, 457, 1904-05.

The muscle juice of the pigeon, however, behaved quite differently; we found it a very energetic reducer, although we have evidence that its power of reduction disappears rapidly after the death of the animal. While we should not place pigeon muscle at the head of a list of tissues arranged in the relative order of their reducing powers, as did Bernstein,<sup>20</sup> yet we agree with the general results of this work in that we place the pectoralis major of the pigeon as second only to its liver in the intensity of reducing power.

While we are not in a position at present to offer a satisfactory explanation of the difference in the reducing power between mammalian and avian muscle juices, we can appreciate the physiological desirability of such a muscle as the bird's pectoralis major being able with the utmost rapidity to obtain from its blood the maximal quantity of oxygen; in other words, to reduce that blood perfectly in as short a time as possible.

The heart muscle juice (cat) was, as compared with liver juice (cat), a feeble reducer. Although there was some haziness observed between the two bands of oxyhemoglobin, reduction was never complete as judged by our spectroscopic standard. Cat cardiac muscle, therefore, falls into the same category as regards reduction as the striated body muscle of the cat. Of course, the same remark applies to this substance; namely, that in it there are energy transformations of the same order as are going on in voluntary muscle. As judged by direct measurements, Barcroft<sup>21</sup> has shown that the heart muscle has only one-third the gaseous metabolism of the kidney. It may be further remarked that the hearts used were all from kittens; that is, immature animals in which it is well known to physiologists that tissue metabolism is less intense than in adult. The fact that

<sup>20</sup> Bernstein's work is thus described in Schäfer's *Text Book of Physiology*, i, p. 782, Edinburgh and London, 1898: "Tissues placed in normal saline containing haemoglobin quickly reduce that substance, and in this respect muscle is the most effective. Bernstein found the following values for the rate of reduction: muscle 100; liver 81.47; involuntary muscle 72.4; and the mucous membrane of the stomach 57.05" . . . . "This relative power of reduction holds good for tissues taken from frogs and from mammals." (Bernstein, *Untersuch. a. d. physiol. Inst. d. Univ. Halle*, i, p. 107, 1888.)

<sup>21</sup> J. Barcroft and W. E. Dixon: *Jour. Physiol.*, xxxv, p. 203, 1906-07.



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the kidneys were also from kittens (immature animals) has to be remembered in connection with the relatively long time (fifty-seven minutes) taken by the renal reductase to effect complete reduction of the oxyhemoglobin in contact with it at 40°. It is well known that immature animals take a much longer time to drown than do adult; in terms of tissue respiration this may be stated as due to a less intense oxidation of the tissues and, therefore, a less rapid reduction of the blood supplying them. That there is some probability in our surmise is supported by Vernon's<sup>22</sup> work on adult cat tissues and on kitten's. Speaking of the intracellular ferment crepsin he writes: "In the more active cat we find that the muscles of the adult contain twice as much ferment as those of a young kitten." But apart altogether from the age of the animal, the kidney of the cat does not rank as an active reducer. Judged by our criteria, the tissues of kidney are not in the same class as the liver as reducing agents. This finding would be in agreement with the statement by Barcroft: "Normally the blood leaving the kidney contains more than 60 per cent of its oxygen;" and again, "Blood . . . of the kidney is very arterial."<sup>23</sup>

As far as our experience has gone, liver juice is the most active reducing agent we have examined, and that of the pigeon the most active of all types of liver. This would seem to be in agreement with the conclusion<sup>24</sup> of Batelli and Stern working with tissue catalase. The late Prof. C. A. Herter, in his Harvey lecture (1906), in speaking of anaerobic conditions in bacteriological research said: "The reducing action of fresh liver has been successfully employed by Prof. Theobald Smith in rendering the closed arm of the fermentation tube more strictly anaerobic."

We have found reductase in the tissue of animals of four of the five great groups of the Vertebrata; namely, mammals, birds, amphibia, and fishes. The reductase of fish liver we found exceedingly powerful both as regards oxyhemoglobin and soluble Prussian blue. It reduced cat blood as rapidly as it did fish blood. Its energy of reduction is very remarkable when we consider that at the time of our examination of the liver of the fish, it had been

<sup>22</sup> H. M. Vernon: *Jour. Physiol.*, xxxiii, p. 85, 1905-06.

<sup>23</sup> J. Barcroft and M. Camis: *Jour. Physiol.*, xxxix, p. 134, 1909-10.

<sup>24</sup> M. F. Batelli and L. Stern: *Arch. d. fisiol.*, ii, p. 471, 1905.

dead for about twenty-four hours. The physiological advantage to the fish to have a powerful reducer in its tissues is too obvious to be more than mentioned. Seeing that fish have at their disposal relatively such small amounts of oxygen, it is of the highest consequence to them to be able to extract it from their blood with the greatest possible thoroughness.

The biological significance of there being no specificity of interaction between the reductase of one animal and the oxyhemoglobin of another, is that the oxyhemoglobin in the blood of A can be reduced by the reductase of B, A and B being in widely separated groups. Thus if human reductase were unable to reduce a foreign oxyhemoglobin, the perfusion into man of the blood of a lower animal would be absolutely useless in that the tissues could not cause that oxyhemoglobin to dissociate and so would be asphyxiated. It is, however, well known that when danger arises from the introduction of a foreign blood, it is not due to any difficulty about its being reduced, but to the liability of the human erythrocytes being hemolyzed by the blood of the lower animal. That the reductase of any animal can reduce the oxyhemoglobin of any other might be expected from the uniformity of composition of hemoglobin throughout the animal kingdom. As far as reductase is concerned, there is no "specificity" of hemoglobin in the sense of Bradley and Sansum,<sup>25</sup> who used the anaphylactic reaction of the guinea pig to demonstrate it.

We think that we may venture, in conclusion, to make a suggestion in connection with the hypothesis of the mechanism of tissue respiration which is given by Vernon and is based on Dakin's. Vernon writes:<sup>26</sup>

The tissues contain a substance which can absorb oxygen from their surroundings to form an organic peroxide, and by the help of a peroxidase can transfer this oxygen to amino acid and carbohydrate molecules bound up in the tissues. . . . The organic peroxide, though it can still effect some oxidation, cannot of itself carry it to the final carbon dioxide stage.

It seems to us that Vernon's "substance which can absorb oxygen" is none other than the tissue reductase whose behavior we have been studying. In the earlier part of this investigation,<sup>27</sup>

<sup>25</sup> H. C. Bradley and W. D. Sansum: *this Journal*, xviii, p. 497, 1914.

<sup>26</sup> Vernon: *Jour. Physiol.*, xxxix, p. 182, 1909-10.

<sup>27</sup> Harris and Creighton: *Proc. Roy. Soc., Series B*, lxxxv, p. 487, 1912.

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we pointed out that reductase could not only reduce compounds containing oxygen, such as methemoglobin and sodium nitrate, but such relatively stable substances containing no oxygen, as ferric chloride and soluble Prussian blue. In other words, the living tissues contain a substance with energetic reducing powers; we have brought forward a good deal of new evidence that this substance is an intracellular enzyme, which has been named reductase. It must be reckoned with in any hypothesis of tissue respiration. Starling<sup>28</sup> does, indeed, to some extent do this: "There is no doubt that reducing substances are formed under normal circumstances in the tissues, as is shown by the methylene blue experiment, and it is possible that such reducing substances may aid in activating oxygen and in the induction of certain oxidative processes." Our work enables us to be more definite in regard to these "reducing substances;" it seems to warrant our asserting that the reductions effected by living tissues and by their juices *in vitro* are carried out by an endo-enzyme, already named reductase, some of whose properties we have investigated. It is not enough to describe the activity of an oxidase in this connection; our observations have shown that the tissues are energetic reducers on account of the presence in them of an enzyme, some of whose properties and behavior we have elucidated. If tissue respiration is completed by an oxidase, *it is originated by reductase.*

### V. SUMMARY.

1. The reduction of oxyhemoglobin, at 40°, to the one-banded condition by the reductase contained in the liver and certain other organs of mammals, birds, amphibia, and fish has been investigated spectroscopically.

2. It has been found that the liver juice of all these classes of animals has the greatest reducing power, that of the pigeon being the most active of all the animals studied; the muscle juice of the cat is but a feeble reducer; while, on the other hand, the muscle juice of the pigeon is only second in activity to its liver juice. Press juices of kidney, stomach, pancreas, heart, and cortex cerebri exhibit varying degrees of activity.

<sup>28</sup> E. H. Starling: *Principles of Human Physiology*, London, 1912, p. 123.

3. In addition to reducing oxyhemoglobin, the press juices of the organs studied reduce soluble Prussian blue to the leuco compound.

4. It has been found that there is no specificity between reductase and oxyhemoglobin, in that the reductase of any one animal can reduce the blood of any other.

5. The influence of temperature on the rate of reduction of oxyhemoglobin by the reductase contained in cat liver juice has been investigated, and it has been found that the rate of reduction increases with rise of temperature. Between 10° and 40° the temperature coefficient of the process of reduction is approximately 2. Above 40° its value rapidly decreases with rise in temperature. Below 10° the rate of reduction is rapidly retarded, until below 0° inhibition of the enzyme is practically complete. Liver juice which has been kept below 0° for a time regains its original activity on raising its temperature.

6. It has been shown that the activity of the reductase of cat liver juice decreases with the age of the juice, becoming practically inactive at the end of about eight days. The curves obtained by plotting the reducing power of the liver juice against the age of the juice are logarithmic. A kinetic investigation of the decay in the activity of reductase shows that the process follows the unimolecular law.

7. The physiological bearing of the results of the investigation has been discussed. It is contended that reductase is the chief factor in causing dissociation of oxygen from oxyhemoglobin (reduction) in the tissues; carbon dioxide, traces of acid, and temperature being all of subsidiary importance. These observations tend, as was hoped, to throw some light on the biochemical aspect of the inspiratory phase of internal respiration, the process whereby oxygen is removed from the oxyhemoglobin and from the lymph bathing the living cells, and applied in the nascent state to oxidisable substances in the cells.

In conclusion, the bearing of the results of this investigation upon the process of tissue respiration has been discussed, and it has been shown that if tissue respiration is completed by an oxidase, it is originated by reductase. No statement as to the physicochemical rationale of tissue respiration is complete which does not recognize the existence and capabilities of reductase.



## STUDIES IN CARBOHYDRATE METABOLISM.

### VIII. THE INFLUENCE OF HYDRAZINE ON THE UTILIZATION OF DEXTROSE.

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A long series of investigations has shown beyond question that the amount of dextrose in the blood varies little throughout normal life. No great differences are found even in the blood of different species; and it may be said in general that the percentage of sugar in the blood is one of the body constants. This, of course, does not apply to pathological conditions, and for that reason natural and artificial abnormalities have been carefully observed. As knowledge has accumulated, a number of procedures have been made available for producing variations in the blood picture, with respect to the sugar content. Most of these increase the amount of glucose, but by a few methods a condition of hypoglycemia can be obtained. One of the most effective agents in producing the latter condition is hydrazine, which was the medium of attack in this investigation.

The earlier literature concerning the action of this compound is very scanty, though the toxicity of hydrazine has long been known. A complete account of the earlier papers is found in the recent publications of Underhill and his pupils, who have demonstrated the influence of hydrazine poisoning upon the sugar content of the blood. Underhill and Kleiner<sup>2</sup> showed in the first paper on the subject that the partition of the nitrogenous constituents in the urine was only slightly altered by the administration of moderate doses of hydrazine. The essential positive finding was that the poison exerts a selective action on the liver, as indicated by fatty

<sup>1</sup>The essential facts in this paper are taken from the dissertation presented by Albert G. Hogan for the degree of Doctor of Philosophy, Yale University, 1914.

<sup>2</sup>F. P. Underhill and I. S. Kleiner: this *Journal*, iv, p. 165, 1908.

infiltration. In a later research<sup>3</sup> a quantitative study of the blood was made, and it was discovered that one of the typical effects was a marked hypoglycemia. This condition was very constant in dogs; rabbits were more variable. A striking fact that came out in this study was the action of dextrose on dogs that had received hydrazine. The sugar when given subcutaneously in doses of 5 gm. per kilo promptly caused the death of the animal. This work was followed<sup>4</sup> by an investigation of the relation of hydrazine to pancreatic diabetes. It was demonstrated at this time that the glycosuria which follows removal of the pancreas can be temporarily inhibited by moderate doses of hydrazine.

The facts that are now known concerning the toxicity of hydrazine fail completely to afford any explanation of its action. If, however, the processes here involved were once made clear, it is probable that valuable information on the intermediary metabolism of carbohydrates would be available. In this connection recent work of Frank and Isaac<sup>5</sup> deserves consideration. They poisoned animals with phosphorus, which in some respects resembles hydrazine in its action. It causes hypoglycemia and the rapid disappearance of glycogen. In both cases, also, the liver shows a characteristic yellow color, presumably due to fatty infiltration. As was pointed out in the paper of Underhill and Kleiner, however, there are some essential differences in the action of these two poisons. Hydrazine limits its action to the cells of the liver and affects only the cytoplasm; the cells first attacked are in the center of the lobules. In phosphorus poisoning the periphery of the liver seems to be injured first. It attacks the cell nuclei and does not limit its action to that organ. Frank and Isaac in their study of phosphorus poisoning devoted considerable attention to the sugar of the blood. They administered dextrose to rabbits under different conditions, and observed the time necessary for the blood sugar content to become normal. The sugar was administered through a stomach sound in doses of about 10 grams per kilo. When given to a normal rabbit the blood sugar content increased to about 0.3 per cent and became normal again in about four hours. When the dextrose was given

<sup>3</sup> F. P. Underhill: this *Journal*, x, p. 159, 1911-12.

<sup>4</sup> F. P. Underhill and M. S. Fine: this *Journal*, x, p. 271, 1911-12.

See also Underhill: this *Journal*, xvii, pp. 293 and 295, 1914. F. P. Underhill and A. L. Prince: this *Journal*, xvii, p. 299, 1914.

<sup>5</sup> E. Frank and S. Isaac: *Arch. f. exper. Path. u. Pharmacol.*, lxiv, p. 274, 1911.

shortly after the phosphorus had been administered, the course of the blood sugar closely followed the normal curve. If the interval between the introduction of phosphorus and dextrose was increased to twenty-four hours, however, then the abnormally high sugar content was reduced very slowly, being delayed ten hours in some cases. Since phosphorus produces results so similar to hydrazine in some respects, and so unlike it in others, it seemed desirable to make similar trials with the latter drug.

#### EXPERIMENTAL.

*Plan.* The plan adopted was to administer hydrazine to an animal, and at the time when its effects were most evident to introduce dextrose subcutaneously. The result of such a subcutaneous injection both in a normal and in a poisoned animal is to raise the blood sugar content. Underhill showed that hydrazine may cause marked hypoglycemia in rabbits, a finding that was confirmed in this investigation. If this reduction is due to some increase in the glycolytic processes, it would seem probable that dextrose administered in large quantities would be promptly disposed of, more quickly than by a normal rabbit.

*Methods.* The experimental animals used were rabbits. The general plan was to administer the maximum non-fatal dose of hydrazine, and then to make subcutaneous injections of dextrose at the time when the sugar content of the blood was at its minimum. The method of determining the blood sugar was that devised by Forschbach and Severin.<sup>6</sup> Before attempting the above experiments it seemed desirable to determine the optimum experimental conditions; as, for example, dosage of hydrazine and the amount of dextrose to be injected. Other factors involved were the effect of starvation, as animals will not eat after the administration of hydrazine, and the normal time required for the utilization of the amount of dextrose to be introduced.

#### *The optimum conditions for the production of hydrazine hypoglycemia in rabbits.*

The first point established was the most suitable dosage of hydrazine for the production of hypoglycemia, together with the

<sup>6</sup> Forschbach and Severin: *Arch. f. exper. Path. u. Pharmacol.*, lxxviii, p. 341, 1912.



time required for it to exert its action. Hydrazine sulphate (Kahlbaum's) in 2.5 per cent solution was administered subcutaneously; the dose varied from 60 to 85 mgm. per kilo of body weight. At various intervals the per cent of dextrose in the blood was determined, in order to find what dosage was most effective, and how much time elapsed after its administration before the blood sugar reached its lowest value. The general method for obtaining blood was to fasten the animal on a rabbit board in a horizontal position, and make a transverse incision in the marginal ear vein. The blood was allowed to fall directly into the weighing bottle; about twenty or thirty drops are required. If the blood flows too slowly it can be hastened by warming the ear with an electric light bulb. Table I contains the experimental data.

*The influence of inanition on the sugar content of the blood.*

Inasmuch as rabbits refuse food when under the influence of hydrazine, it became essential to determine the influence of starvation alone upon the blood sugar content. The general plan adopted was similar to that given above. The animals received no food, and the blood sugar was determined daily through a period of approximately one week. The results are given in Table II.

The results in this table indicate that starvation does not influence the per cent of sugar in the blood.

*The rate of disappearance from the blood of glucose subcutaneously injected into starving rabbits.*

In order to formulate a satisfactory working basis, a series of determinations in normal animals was made to show how rapidly a subcutaneous administration of glucose would disappear. This was done by injecting various amounts of sugar and examining the blood at frequent intervals. Food was withheld from the rabbits for a period of two days before injecting the dextrose. For the results see Table III.

These experiments demonstrate that if 3 to 5 grams of dextrose are administered subcutaneously to normal rabbits the percentage of blood sugar usually regains the normal within a period of three to four hours.

*The amount of hydrazine required to produce hypoglycemia in rabbits, and the time necessary for the drug to exert its action.*

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*The rate of disappearance from the blood of glucose subcutaneously injected into hydrazinized rabbits.*

Finally determinations were made to find how rapidly dextrose disappeared following administrations of hydrazine. In this series the normal percentage of blood sugar was obtained, and then hydrazine was given. Two days later the blood sugar content was again estimated. The dextrose was then administered

TABLE II.

*The effect of inanition on the per cent of sugar in the blood.*

NO. OF ANIMAL	PER CENT OF SUGAR IN THE BLOOD			
	Normal	After a starvation period of		
		2 dys.	3 dys.	4 dys.
17	0.12	0.12	0.15	0.12
18	0.12	0.12	0.11	0.12
19	0.10	0.10	0.14	0.11

TABLE III.

*The content of blood sugar at intervals after subcutaneous injections of dextrose.*

NO. OF ANIMAL.....	20	21	22	23	27	28	29
WEIGHT IN KILOS.....	1.7	1.2	1.8	1.4	1.9	1.6	1.8
GM. OF DEXTROSE PER KILO INJECTED.....							
IN 30 PER CENT SOLUTION	5	5	5	5	5		
IN 15 PER CENT SOLUTION						1.5	3.0
PER CENT OF BLOOD SUGAR NORMAL.....	0.12	0.17	0.11	0.13	0.18	0.09	
AFTER INJECTING DEXTROSE..							
" $\frac{1}{4}$ HR. ....		0.33	0.30	0.28			
" $\frac{1}{2}$ " .....	0.22	0.34	0.28	0.36	0.31	0.19	0.33
" $\frac{3}{4}$ " .....	0.27	0.33	0.31	0.35			
" 1 " .....	0.30	0.33	0.35	0.36	0.46	0.09	0.33
" $1\frac{1}{2}$ " .....	0.31	0.25	0.24	0.38	0.53	0.08	0.28
" 2 " .....	0.25	0.18	0.20	0.31	0.48	0.08	0.28
" $2\frac{1}{2}$ " .....	0.30	0.15	0.20	0.25	0.47	0.12	0.23
" 3 " .....	0.25	0.07	0.10	0.20	0.40	0.09	0.18
" $3\frac{1}{2}$ " .....		0.09	0.14	0.18	0.40		0.15
" 4 " .....		0.13	0.15	0.22	0.39		0.14
" $4\frac{1}{2}$ " .....		0.16	0.15	0.20	0.36		
" 5 " .....			0.15	0.24	0.30		
" 6 " .....	0.13				0.30		

TABLE IV.  
*The effect of hydrazine poisoning on the rate of utilization of dextrose.*

NO. OF ANIMAL.....	24	25	26	30	31	32	33	34	35	36
WEIGHT IN KILOS.....	2.4	1.3	1.5	1.8	1.3	1.4	1.2	1.2	1.5	1.3
MGM. OF HYDRAZINE PER KILO INJECTED.....	75	75	75	75	75	75	80	80	80	80
GM. OF DEXTROSE PER KILO INJECTED.....	5*	3*	2.5*	3*	3*	3*	3*	3*	3*	3*
INTERVAL IN DYS. BETWEEN INJECTION OF HYDRAZINE AND DEXTROSE.....	2	3	2	2	1	1	2	3	3	1
BLOOD SUGAR, NORMAL, PER CENT.....	0.11		0.11	0.12	0.13	0.13	0.11	0.10	0.14	0.10
BEFORE INJECTING DEXTROSE.....	0.16	0.14	0.04	0.10	0.07	0.07	0.11	0.10	0.14	0.10
AFTER INJECTING DEXTROSE										
" 1 HR.....	0.33	0.24								
" 1 1/2 ".....	0.48	0.29	0.27	0.25						
" 3 ".....	0.56	0.35								
" 1 ".....	0.66	0.41	0.34	0.32						
" 1 1/2 ".....	0.66	0.32	0.41	0.33						
" 2 ".....	0.33	0.32	0.39	0.27	0.37	0.33	0.25	0.41	0.37	0.28
" 2 1/2 ".....	0.37	0.32	0.45	0.34						
" 3 ".....	0.43	0.27	0.37	0.38						
" 3 1/2 ".....	0.65	0.28		0.41						
" 4 ".....	0.62	0.26	0.39	0.19	0.27	0.16	0.20	0.24	0.22	
" 4 1/2 ".....	0.65	0.27	0.38							
" 5 ".....	0.54	0.27								
" 5 1/2 ".....	0.20	0.31								
" 6 ".....	0.30				0.16	0.18		0.10	0.14	0.20
" 7 ".....	0.20									

\* In 30 per cent solution.

# In 15 per cent solution.

subcutaneously, and the amount of sugar in the circulation was determined at varying intervals. The results are given in Table IV.

A comparison of Tables III and IV indicates that hydrazine markedly retards the utilization of dextrose after subcutaneous injections of the sugar.

#### DISCUSSION.

A survey of the data tabulated indicates that, when introduced into rabbits, 80 mgm. of hydrazine sulphate per kilo is probably the largest dose that can be given without lethal effects. Seventy-five mgm. is a safer amount, and perhaps just as effective. It is quite evident, however, that the reaction of the rabbit to hydrazine is very inconstant. While the blood sugar of dogs is reduced to minimal values with a dosage of 50 mgm. per kilo, it is frequently impossible to obtain that result when using rabbits, whatever amount is given. As might be expected, the amount of sugar in the blood during starvation does not vary from normal. Starvation alone, therefore, plays no significant part in the action of hydrazine in respect to the content of blood sugar.

When dextrose is injected subcutaneously into a normal rabbit, there is considerable variation in the rapidity with which it disappears from the circulation. The concentration of the solution probably has some effect on the time required for absorption. A comparison of Tables III and IV shows, however, that if dextrose is subcutaneously administered during hydrazine poisoning, the resumption of a normal blood sugar content is markedly delayed.

#### SUMMARY.

Hydrazine causes hypoglycemia in rabbits as in dogs, but not so consistently.

Starvation causes no change in the content of blood sugar.

When dextrose is administered two days after the administration of hydrazine, its utilization as indicated by the blood sugar content is markedly retarded. This is true whether the amount of sugar in the blood was previously low or not.

These facts offer no explanation for the observed diminution of dextrose in the blood after the administration of hydrazine.

## STUDIES IN CARBOHYDRATE METABOLISM.

### IX. THE INFLUENCE OF HYDRAZINE ON THE GLYOXALASE ACTIVITY OF THE LIVER.

By FRANK P. UNDERHILL AND ALBERT G. HOGAN.<sup>1</sup>

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(Received for publication, January 7, 1915.)

Modern theories of metabolism assign vast importance to enzyme action. These hypotheses, though completely in accord with the facts as determined *in vitro*, should be extended to processes in the body with great caution. In accordance with our present ideas, however, it seems quite possible that the peculiar effect that hydrazine<sup>2</sup> produces on the sugar content of the blood may be due to some interference with the essential enzymatic activities. In this connection three possibilities suggest themselves. The enzyme or enzymes concerned may be accelerated, retarded, or entirely destroyed.

#### EXPERIMENTAL.

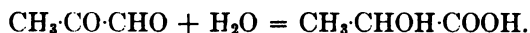
*Problem.* In view of these possibilities the plan adopted for this investigation was to make a series of observations on the influence of hydrazine on the glyoxalase activity of the liver. As Dakin and Dudley,<sup>3</sup> who discovered glyoxalase, have pointed out, it is very active in liver extracts. Furthermore, it is probably directly concerned in the intermediary metabolism of car-

<sup>1</sup> The essential facts of this paper are taken from the dissertation presented by Albert G. Hogan, for the degree of Doctor of Philosophy, Yale University, 1914.

<sup>2</sup> Compare previous paper: this *Journal*, xx, p. 203, 1915.

<sup>3</sup> H. D. Dudley and H. W. Dakin: this *Journal*, xiv, pp. 155, 423, 555, 1913; also xv, pp. 127 and 463, 1913.

bohydrazes. The reaction involved is reversible, and proceeds as follows:



The results of Dakin and Dudley were confirmed and extended by Levene and Meyer.<sup>4</sup> They showed that leucocytes or kidney tissue if allowed to act on methyl glyoxal will convert it to lactic acid. Inasmuch as hexoses are also converted to lactic acid in this way, it is rendered even more probable that methyl glyoxal is an intermediate stage in carbohydrate metabolism. If, therefore, glyoxalase is concerned in this process, it would seem especially adapted to the problem here involved.

*Methods.* Because of its ready response to hydrazine poisoning, the dog was chosen as the experimental animal. The dosage employed was 50 mgm. of hydrazine sulphate (Kahlbaum's) per kilo, and was administered subcutaneously in 2.5 per cent solution. The maximum effect of the compound is usually attained within forty-eight hours; so on the second day after administering the drug the dog was killed. The procedure following was essentially that devised by Dakin and Dudley. The liver was finely ground and a 20 per cent extract prepared with distilled water. This was kept in a thermostat at a temperature of 35° or 37°C. for one hour, and then strained through several layers of muslin cloth. All manipulations were aseptic as far as possible, and no preservatives were used. Putrefaction rarely occurred and seemed to have no appreciable effect on the amount of mandelic acid present. Dakin found that phenyl glyoxal has certain advantages over the methyl compound for experimental purposes, so it<sup>5</sup> was used in this investigation. It undergoes the same type of reaction, and the mandelic acid formed has a much higher specific rotation than lactic acid. A quantitative estimation, therefore, is much more accurate.

The proportions used were 0.2 of a gram of phenyl glyoxal, dissolved in 5 cc. of distilled water, and 50 cc. of the tissue extract. The phenyl glyoxal solution was filtered before use. Freshly

<sup>4</sup> P. A. Levene and G. M. Meyer: this *Journal*, xiv, p. 551, 1913.

<sup>5</sup> We are greatly indebted to Dr. Dakin for supplying us with a large quantity of phenyl glyoxal.

precipitated calcium carbonate was added to the digestion mixture to preserve neutrality. At the end of the incubation period the mixture was removed from the thermostat and boiled. In order to determine the relative amount of mandelic acid, the procedure was as follows: 30 grams of ammonium sulphate were added to the solution and the mixture was heated on the water bath for three minutes. This was cooled and acidified with syrupy phosphoric acid. The precipitate was filtered off and washed with a solution of ammonium sulphate. Three cc. more of the phosphoric acid were added, and the mandelic acid was extracted by shaking out with ether; four extractions are sufficient. The ether extract was washed carefully with small quantities of water to remove traces of phosphoric acid, and then evaporated. The residuum was taken up in a little water, and filtered into a 200 mm. polariscope tube, with a capacity of about 17 cc. The tube was almost completely filled with wash water from the funnel, and after closing it was inverted repeatedly until the contents were thoroughly mixed. After determining the rotation the contents of the tube were transferred to a beaker, and titrated against  $\frac{N}{10}$  sodium hydroxide, with phenolphthalein as an indicator.

Since the speed of the reaction was the point of greatest interest, a number of these estimations were made for each animal. These were incubated for different periods ranging from thirty minutes to nine hours in most cases. One was boiled before adding the phenyl glyoxal, and served as a control. In order to ascertain whether the hydrazine was producing its typical effects, determinations of the blood sugar were made. With one exception the amount was found to be very low. Dakin has demonstrated that pancreas extract inhibits the enzyme glyoxalase, so an experiment was devised to determine whether the pancreas of a hydrazinized animal also exercises this inhibition. Fifty cc. of a 20 per cent pancreas extract were added to 50 cc. of a 20 per cent liver extract of the same animal, which had received hydrazine. A similar mixture was made with a boiled pancreas extract to serve as a control. In a few cases the effect of using only a 10 per cent tissue extract was tried. The results of the various trials are given in the tables.



TABLE I.  
*Normal liver.*

RATE OF GLYOXALASE ACTIVITY										
20 per cent extract										
HRS. INCUBATED	ROTATION					ACIDITY cc. $\frac{N}{10}$ NaOH				
	No. of animal									
	1	2	3	4	5	1	2	3	4	5
1		-2.2° V.	-1.1° V.	-1.7° V.	-1.3° V.		8.00	1.05	2.5	2.2
1		-4.3° V.	-1.9° V.	-2.8° V.	-2.4° V.		9.65	1.8	3.5	3.4
2		-5.4° V.	-2.6° V.	-5.5° V.	-3.7° V.		10.15	2.9	6.6	5.6
3	-5.0° V.	-4.4° V.	-3.1° V.	-5.8° V.	-5.1° V.	10.51	14.55	2.8	7.4	7.1
6	-4.8° V.					9.95				
9	-4.2° V.	-5.4° V.	-5.3° V.	-6.0° V.	-4.1° V.	9.75	10.1	4.7	7.2	7.5
12	-4.5° V.					9.90				
20	-7.5° V.					13.30				
				10 percent extract						
1				-2.5° V.	-0.8° V.				2.4	2.3
2				-2.5° V.	-1.3° V.				3.1	2.9
3				-3.3° V.	-1.3° V.				4.0	2.9
6				-3.3° V.	-2.0° V.				3.8	3.9
		Control.	20 per cent extract		(boiled)					
9	-0.4° V.	0	0	0	0	3.7	2.82	0	0.1	0.45

TABLE II.  
*Hydrazinized liver.*

RATE OF GLYOXALASE ACTIVITY									
20 per cent extract									
HRS. INCUBATED	ROTATION				ACIDITY				
	No. of animal								
	6	7	8	9	6	7	8	9	
1	-1.2° V.	-1.4° V.	-1.2° V.	-1.2° V.	4.5	1.9	1.8	2.2	
1	-2.9° V.	-2.3° V.	-2.6° V.	-2.5° V.	5.2	3.1	3.9	3.6	
2	-4.4° V.	-4.9° V.	-4.5° V.	-4.4° V.	6.6	6.5	5.8	5.4	
3	-4.8° V.	-4.4° V.	-5.7° V.	-4.8° V.	8.1	6.3	7.2	5.7	
6		-5.3° V.	-5.5° V.	-4.3° V.		6.7	8.1	5.8	
12	-5.6° V.				10.0				
20	-4.8° V.				7.7				
Control (9 hrs.)	0	0	-0.2° V.	0	1.1	0.6	1.2	0.6	
			10 per cent extract						
1			-1.1° V.	-0.7° V.			1.6	1.4	
2				-1.4° V.				2.3	
3			-2.0° V.	-2.0° V.			3.0	2.8	
9				-3.0° V.				4.0	

TABLE III.  
*Comparison of results.*  
*Normal dogs.*

NO. OF ANIMAL	ROTATION OBSERVED AFTER INCUBATING				
	$\frac{1}{2}$ hr.	1 hr.	2 hrs.	3 hrs.	9 hrs.
1				-5.0° V.	-4.2° V.
2	-2.2° V.	-4.3° V.	-5.4° V.	-4.4° V.	-5.4° V.
3	-1.1° V.	-1.9° V.	-2.6° V.	-3.1° V.	-5.3° V.
4	-1.7° V.	-2.8° V.	-3.7° V.	-5.8° V.	-6.0° V.
5	-1.3° V.	-2.4° V.	-3.7° V.	-5.1° V.	-4.1° V.
<i>Hydrazinized dogs.</i>					
6	-1.2° V.	-2.9° V.	-4.4° V.	-4.8° V.	
7	-1.4° V.	-2.3° V.	-4.9° V.	-4.4° V.	-5.3° V.
8	-1.2° V.	-2.6° V.	-4.5° V.	-5.7° V.	-5.5° V.
9	-1.2° V.	-2.5° V.	-4.4° V.	-4.8° V.	-4.3° V.

An extract of the pancreas, in the case of Dog 8, was added to the liver extract, and was found to inhibit completely glyoxalase. The unboiled pancreatic extract had no effect whatever.

#### SUMMARY.

A comparison of results indicates that the glyoxalase activity of the liver is not markedly altered by the action of hydrazine.

It is, therefore, evident that the results of this investigation offer no explanation for the disappearance of glycogen from the liver and the diminished blood sugar content observed after hydrazine administration.



## A COLORIMETRIC METHOD FOR THE ESTIMATION OF AMINO-ACID $\alpha$ -NITROGEN.

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(Received for publication, January 14, 1915.)

Two methods are at present in use for the determination of amino-acid nitrogen. The first, that of Sørensen,<sup>1</sup> is a rapid, easy method, but is neither very delicate nor accurate. The second, that of Van Slyke,<sup>2</sup> is the method now usually accepted for the determination of nitrogen in amino groups. In its latest development, the micro chemical form,<sup>3</sup> the method will estimate 0.5 mgm. of nitrogen with an accuracy of 1 per cent, and its application to the determination of amino-acid nitrogen in blood and tissues has yielded in its author's hands very valuable and interesting results. It was felt, however, that a third and independent method for the estimation of the  $\alpha$ -nitrogen of the amino-acids, especially if the sensitiveness of the reaction could be increased beyond that of the Van Slyke method without loss of accuracy, would not only be valuable but necessary as the time approached for a study of the chemistry of the single cell.

It soon became apparent that such a hope could be fulfilled only by the quantitative application of a color reaction of amino-acids, and of all such the most probable seemed the reaction with triketohydrindene hydrate.

This reaction was discovered by Ruhemann,<sup>4</sup> who found that all acids containing a free amino group in the  $\alpha$  position reacted with triketohydrindene hydrate with the production of an intense blue color.  $\beta$ -,  $\gamma$ -, and  $\delta$ -amino-acids only gave small amounts of color, and  $\alpha$ -amino-acids substituted on the amino or carboxyl

<sup>1</sup> S. P. L. Sørensen: *Biochem. Ztschr.*, vii, p. 45, 1908.

<sup>2</sup> D. D. Van Slyke: this *Journal*, ix, p. 185, 1911; xii, p. 275, 1912.

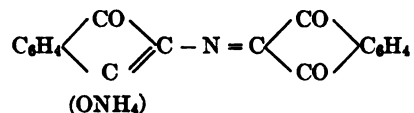
<sup>3</sup> Van Slyke: *ibid.*, xvi, p. 121, 1913-14.

<sup>4</sup> S. Ruhemann: *Tr. Chem. Soc.*, xcvii, pt. ii, p. 2025, 1910.

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group did not react at all. This discovery was confirmed and extended by Abderhalden, who applied it to the detection of pregnancy and cancer, triketohydrindene hydrate now being a commercial product under the name of "ninhydrin." The sensitiveness of the reaction is unquestioned; for, according to Abderhalden and Schmidt,<sup>5</sup> it will detect one part of glycine in 65,000 parts of water, though the other amino-acids will not react in quite so dilute a solution.

The constitution of the blue coloring matter was investigated by Ruhemann,<sup>6</sup> who isolated from the interaction of alanine and triketohydrindene hydrate a body which was found to be identical with the *ammonium* salt of *diketohydrindylidene-diketohydrindamine*,



Thus the chemistry of the coloring matter was well known and the problem became the determination of the conditions under which the grouping



common to all  $\alpha$ -amino-acids would react quantitatively with triketohydrindene hydrate to produce the blue colored ammonium salt of diketohydrindylidene-diketohydrindamine, which could then be compared colorimetrically with a definite amount of coloring matter as standard.

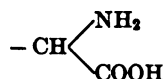
The reaction has already been made use of in a roughly quantitative way by Abderhalden and Lampé<sup>7</sup> in studying the fate of amino-acids during absorption, but they made no attempt to place their results on a strictly quantitative basis. Herzfeld,<sup>8</sup> however, devised a method for the estimation of the group

<sup>5</sup> E. Abderhalden and H. Schmidt: *Ztschr. f. physiol. Chem.*, lxxxv, p. 143, 1913.

<sup>6</sup> Ruhemann: *Tr. Chem. Soc.*, xcix, p. 1486, 1911.

<sup>7</sup> E. Abderhalden and A. E. Lampé: *Ztschr. f. physiol. Chem.*, lxxxi, p. 473, 1912.

<sup>8</sup> E. Herzfeld: *Biochem. Ztschr.*, lix, p. 249, 1914.



using the ninhydrin reaction as a basis. The method was to evaporate the amino-acid and excess of triketohydrindene hydrate to dryness on a water bath, dissolve the purple colored residue in a little alcohol with a drop or two of ammonium hydroxide, make up to a known volume, and determine the amount of coloring matter by measuring its extinction coefficient in a spectrophotometer. The present authors have repeated this method of preparing the coloring matter in a quantitative way, estimating it, however, by a Duboscq colorimeter instead of measuring the extinction coefficient in a spectrophotometer. The former method is much more rapid, and at present is the only one which could be successfully applied in hospital laboratories to the study of amino-acid excretion in pathological conditions. Working in this way, the results were very unsatisfactory. It was found by heating varying amounts of an amino-acid with an excess of ninhydrin that the different amounts of the amino-acid could be estimated with moderate accuracy, using a fixed amount of the same amino-acid as standard. The following figures illustrate this point.

AMINO-ACID	CC. OF 0.1 PER CENT SOLUTION	COLORIMETER READING	
		Found:	Calculated:
Glycine.....	1.0 Standard	Set at 2.00 cm.	
	1.5	1.51	1.33
	2.0	1.00	1.00
Alanine.....	1.0 Standard	Set at 2.00 cm.	
	2.0	0.99	1.00
	4.0	0.48	0.55
Aspartic acid.....	1.0 Standard	Set at 3.00 cm.	
	2.0	1.51	1.50
	3.0	0.95	1.00
Glutaminic acid.....	1.0 Standard	Set at 2.00 cm.	
	1.5	1.33	1.33
	3.0	0.69	0.66

Thus it will be seen that varying amounts of glycine can be estimated by using the color produced by a known amount of

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glycine as standard. The same is true for alanine, and aspartic and glutaminic acids.

When, however, we attempted to estimate alanine, or aspartic or glutaminic acids, using glycine as a standard, the method gave totally erroneous results.

AMINO-ACID	N <sub>2</sub> PER CC.	
	Found:	Calculated:
	mgm.	mgm.
Alanine.....	0.016	0.036
Glutaminic acid.....	0.152	0.096

In the case of aspartic acid the color produced by the reaction was of such a pronounced reddish shade that it was found impossible to match it against the bluish violet color of the standard.

Thus it will be seen that this method of estimating amino-acid nitrogen fails completely.

### *Reaction of amino-acids and triketohydrindene hydrate in slight excess.*

In our earlier experiments on this subject, attempts were made to study the reaction between various amino-acids and the triketone, with strictly molecular amounts of the two substances, but, owing to causes at present unknown, these experiments failed to give any concordant results. Neither of us, working alone or together, could be certain of obtaining any two series of experiments which agreed within 5 per cent. What we desired to do was to heat a known amount of an amino-acid, usually glycine or alanine, with the molecular equivalent of triketohydrindene hydrate and determine the time at which the maximum development of the blue coloring matter took place. This could be accomplished by immersing a series of test-tubes containing the equivalent amounts in a boiling constant-level water bath, removing them one by one at stated intervals (usually five minutes), diluting them to a known volume (100 cc.) with distilled water, using the color produced in the test-tube first removed as standard, and comparing the others against that in a Duboscq colorimeter. At first the comparisons were made by daylight, but it was soon found that much

more consistent readings were obtained by the use of a 25 watt tungsten lamp as a source of illumination in a dark room. The lamp was placed in a conical, semi-opaque shade, the outer end of which was covered with a sheet of tissue paper to diffuse the light; and the whole was placed about a foot away from the colorimeter. In this way a strongly and evenly illuminated field was obtained on the white reflector of the colorimeter only, and thus the eye was free from any other disturbing sources of light during the determinations. A small electric bulb on a convenient switch enabled one to take the vernier readings. In artificial light the bluish colored solution of the coloring matter changes to a red violet, resembling very much the appearance of a dilute solution of potassium permanganate when viewed in daylight.

Even under these conditions, which greatly improved our ability to make concordant readings, the results continued to be very irregular and capricious, when using equimolecular amounts of the two reagents. The presence of traces of impurity, the rapid fading of the coloring matter, and the sensitiveness of the reaction to heat, probably were all factors, not under these conditions properly controlled, which contributed to the failure of our experiments from a quantitative point of view. However, under more stable and more accurately defined conditions, it is hoped at a later date to return to the study of the reaction in equimolecular solution and to endeavor to obtain from it some insight into the mechanism of what is without doubt a complicated series of reactions.

It was soon found, however, that a slight excess of triketohydrindene hydrate enabled us to approach a solution of our problem of the time of the maximum development of the coloring matter in dilute solution. The same method of procedure was adopted as that previously described. The coloring matter in the test-tube first removed was used as standard and the others were read against it. The series of curves in Figure 1 shows the relative amounts of coloring matter plotted against the time, each amino-acid being taken separately.

It will be noticed that the time of the development of the maximum amount of color is different for each amino-acid, thus precluding the use of this technique for a method of estimating amino-acid  $\alpha$ -nitrogen in a mixture of amino-acids. No attempt



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was made to compare one amino-acid against another, as such a series of experiments would have served no useful purpose at that time. Moreover, the actual amounts of color produced in the cases of aspartic and glutaminic acids and asparagine were so small that the reaction liquid was only diluted to 50 cc. instead of 100 cc. Also the color produced by aspartic acid and asparagine was of a pronounced yellowish tint and could not be compared with the reddish violet given by alanine or glycine.

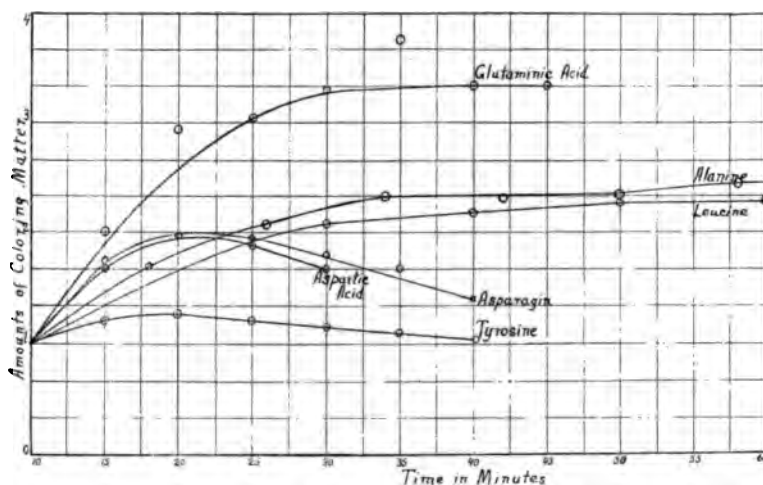


FIG. 1.

### *Reaction of amino-acids and triketohydrindene hydrate in large excess.*

In our next series of experiments we determined the time of the maximum development of color between the different amino-acids and the triketone, using a large excess of the latter in high concentration. The necessity of a high concentration to produce quantitative amounts of color in this reaction had been pointed out by Herzfeld,<sup>9</sup> and a series of experiments of our own had confirmed this conclusion. We heated together in a boiling constant-level water bath a series of 1 cc. of 0.1 per cent solution of amino-acid and 0.5 cc. of 1 per cent solution of triketohydrindene hydrate

<sup>9</sup> Herzfeld: *loc cit.*

for periods of 5, 10, 15, and 20 minutes. The amount of color produced in the test-tube heated for five minutes was taken as standard, and the results were plotted as before. Each amino-acid was considered separately (Figure 2).

The effect of the higher concentration of the triketone was at once readily apparent. The amounts of color produced in the same time were much larger, and concordant experiments were

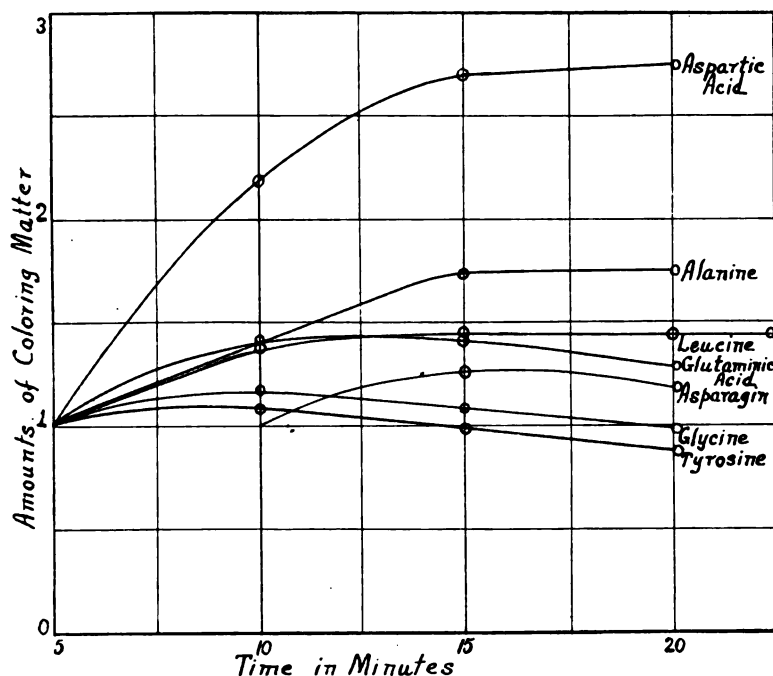


FIG. 2.

easily obtained except in the case of asparagine. It will also at once be noticed that the times of maximum development of color with the different amino-acids are much shorter than in the first series of experiments and lie closer together. Thus, in the first series, the times vary from twenty minutes (tyrosine) to seventy minutes (alanine), whereas in the second series the variation is only from ten minutes (glycine) to twenty minutes (aspartic acid and tyrosine). The other amino-acids possess the time of maxi-

imum development of color at fifteen minutes. The colors with aspartic acid and asparagine, however, were still very weak and still possessed a strong yellowish tinge which rendered their comparison with the other acids extremely difficult. Other irregularities, too, were noticeable. The colors produced by aspartic and glutaminic acids faded very much more rapidly than those produced by the other amino-acids; that produced by aspartic acid being discharged completely in a dark room at the end of twelve hours. As these two acids differed from the others only in the presence of a second carboxyl group, thus rendering them more acidic in character, the disappearance of the coloring matter on standing was put down to this cause. Ruhemann had pointed out that long contact with acids causes a decomposition of the coloring matter, giving a colorless solution, and experiments by us had proved the strong inhibitory effect of small amounts of organic acids on the production of the ammonium salt of diketohydrindylidene-diketohydrindamine from the interaction of alanine and the triketone.

In order to test this assumption a third series of experiments was carried out in presence of a base which would neutralize the acidity of the second carboxyl group of aspartic and glutaminic acids. Such a base should not be strong enough to hydrolyze the triketohydrindene hydrate, as happens with the hydroxides of the alkali metals, and should not interfere with the reductions and condensations which take place in the reaction. The base chosen was pyridine.

*Interaction of amino-acids and triketohydrindene hydrate in large excess, in presence of pyridine.*

One cc. of a 0.1 per cent solution of the amino-acid was added to 0.5 cc. of a 1 per cent solution of triketohydrindene hydrate, and 0.2 cc. of pure, freshly distilled pyridine was added, and the mixture heated in a boiling water bath for varying intervals of time, as in the two previous series of experiments. The estimations of the relative amounts of coloring matter were carried out in a manner similar to the former experiments, each acid being compared with itself only. The curves in Figure 3 show the results obtained. They show quite clearly that the addition of

the pyridine had achieved the desired result. They show that the reaction between amino-acids and triketohydrindene hydrate in presence of pyridine takes place rapidly, and reaches a maximum amount of coloration which remains constant for some minutes, except in the case of aspartic acid. This time of maximum development of color is constant at about twenty minutes. Moreover, the addition of the pyridine had increased enormously the actual amounts of coloring matter produced, and the colors now did not fade appreciably in a short period of time. In the curves obtained with glycine and alanine 0.5 cc. of pyridine was used.

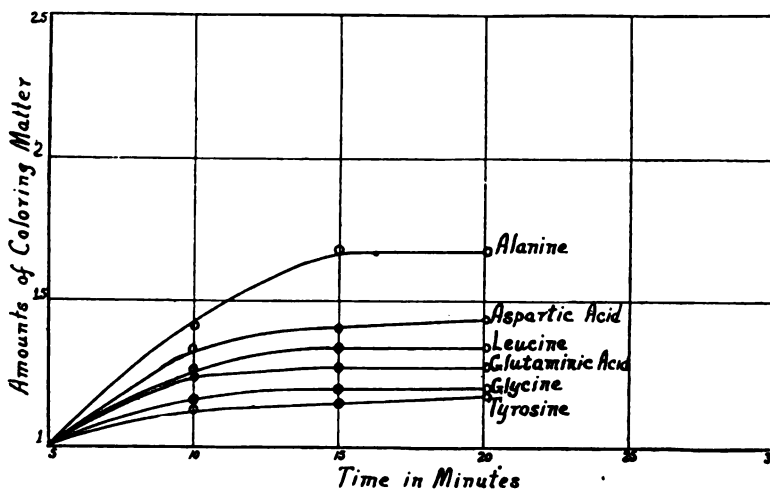


FIG. 3.

A determination was now made of the relative amounts of coloring matter produced by four of the amino-acids, of whose purity we were quite certain, by dissolving equivalent amounts of each of them in water and using one as a standard. The following table will illustrate the experiment and the result. Being in equivalent amounts, they should produce the same amount of coloring matter and thus give the same colorimeter reading.

It will be seen that aspartic acid was still a little low in amount of coloring matter produced when compared with the other acids.

As a result of further experiments, it was decided to reduce the amount of amino-acid nitrogen per cc. to 0.05 mgm., to reduce

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AMINO-ACID	N <sub>2</sub> PER CC.	PYRIDINE (PURE)	TRIKETONE 1.0 PER CENT	COLORIMETER READING
	<i>mgm.</i>	<i>cc.</i>	<i>cc.</i>	
Alanine.....	0.1	0.2	0.5	1.00
Glycine.....	0.1	0.2	0.5	0.99
Aspartic acid.....	0.1	0.2	0.5	1.17
Glutaminic acid.....	0.1	0.2	0.5	1.01

the pyridine to 1.0 cc. of a 10 per cent solution in water, and to increase the triketohydrindene hydrate to 1.0 cc. of a 2 per cent solution.

*Method of estimation of amino-acid  $\alpha$ -nitrogen by use of triketohydrindene hydrate.*

One cc. of the solution to be estimated, containing not more than 0.05 mgm. of amino-acid  $\alpha$ -nitrogen and neutral to phenolphthalein, is mixed with 1 cc. of a 10 per cent aqueous solution of pure pyridine and 1 cc. of a freshly prepared 2 per cent solution of triketohydrindene hydrate and heated in a rapidly boiling constant-level water bath for twenty minutes. At the end of that time the test-tube is removed, cooled, and diluted to a suitable volume, usually 100 cc., but if the amino-acid  $\alpha$ -nitrogen is very small in amount, a correspondingly smaller dilution can be used. The solution of coloring matter thus obtained is compared with the standard color in the usual way, in a Duboscq colorimeter.

*Preparation of a standard color.*

The standard solution is prepared by dissolving 0.3178 of a gram of pure freshly crystallized alanine in a liter of distilled water. Such a solution contains 0.05 mgm. of nitrogen per cc. To prepare the standard color 1 cc. of the standard alanine solution is heated for twenty minutes in a boiling water bath with 0.5 cc. of a 10 per cent solution of pyridine and 1.0 cc. of a 1 per cent solution of triketohydrindene hydrate.<sup>10</sup> At the end of that time the contents of the tube are cooled and diluted to 100 cc. This solution of coloring matter is used as a standard and will keep for twenty-four hours. The standard solution of alanine is

<sup>10</sup> The employment of a more concentrated solution of the ketone gives no further increase in the amount of coloring matter in the case of alanine.

stable for three months. Attempts were made to prepare a permanent standard color without success. A preparation of the pure coloring matter was made according to the directions of Ruhemann, and a dilute solution of it made in water. It was found, however, that when the dried coloring matter was used, it was not as freely soluble in water as the freshly prepared substance, and that an aqueous solution of the pure coloring matter faded rapidly; it was much more stable in the presence of pyridine, but even then at the end of three months it had faded completely. There was thus no advantage to be gained in using a solution of the coloring matter prepared *per se*. The standard color prepared as directed is little or no trouble to prepare along with the determinations themselves.

*Determination of amino-acid  $\alpha$ -nitrogen in various amino-acids and comparison with the method of Van Slyke.*

A series of determinations was then made of the amino-acid nitrogen in various amino-acids, and the results were compared with theory in the case of the pure acid. A parallel series of determinations was made by the method of Van Slyke and the results were compared with the colorimetric determinations. In the case of the acids whose purity was doubtful, the method of Van Slyke served as a standard. The results are expressed in mgm. of nitrogen per cc.

AMINO-ACID	NITROGEN PER CC.		
	Theory	Colorimeter	Van Slyke
	mgm.	mgm.	mgm.
Glycine.....	0.186	0.189	0.189
Alanine.....	0.100	0.101	0.102
Valine.....	0.201	0.201	0.196
Leucine.....		0.104	0.102
$\alpha$ -Amido- <i>n</i> -caproic acid.....	0.160	0.164	0.161
Phenyl alanine.....	0.128	0.126	0.126
<i>i</i> -Tyrosine.....		0.084	0.087
Tryptophane.....		0.090	0.092
Histidine.....		0.085	0.089
Aspartic acid.....	0.105	0.103	0.104
Asparagine.....	0.175	0.178	
Glutaminic acid.....	0.158	0.157	0.157

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The results show quite clearly the excellent agreement of the colorimetric method with theory and with the method of Van Slyke. It must be pointed out that the solutions of the amino-acids were made of just sufficient concentration to be estimated in the Van Slyke micro apparatus. To determine the amount colorimetrically, it was necessary to dilute those solutions from two to four times, so that our actual error of determination is less than appears in the table.

The estimation of cystine, however, by this method gives results which are much too low. Moreover, the coloration developed has an intense red color, which makes it almost impossible to compare it with the standard alanine. As a consequence of this it would be impossible to estimate the amino-acid  $\alpha$ -nitrogen in a mixture of amino-acids containing a large proportion of cystine without a large error. Such cases, however, are not very common, and the authors do not think that the amount of cystine, occurring in the hydrolysis mixture obtained from the majority of proteins, will introduce any serious error. To determine this point and to see if the method would estimate the  $\alpha$ -nitrogen in a mixture of amino-acids in the proportions in which they occur in a native protein, an estimation of the amino-acid  $\alpha$ -nitrogen in ereptone was made and compared with the Van Slyke method.

	N <sub>2</sub> PER CC.	N <sub>2</sub>
	<i>mgm.</i>	<i>per cent</i>
Colorimeter.....	0.132	8.5
Van Slyke.....	0.139	9.0

It will be seen that the colorimetric method gives figures slightly lower than the Van Slyke method, but not low enough to invalidate seriously the result. The difference, however, is capable of easy explanation. The colorimetric method gives too low a result in the presence of cystine. The Van Slyke method gives a result a little too high in the case of glycine and cystine. These three facts are sufficient, we think, to explain the difference of 0.5 per cent.

A series of determinations of the amino-acid  $\alpha$ -nitrogen in peptones from various sources was next carried out in order to

test the efficacy of the method when dealing with partial hydrolysis products of proteins. The following table shows the results to be in good agreement with those obtained by the Van Slyke method.

ORIGIN OF PEPTONE	N <sub>2</sub> PER CC.	
	Colorimeter	Van Slyke
Meat (Merck).....	0.062	0.061
"E carne" (Schuchardt).....	0.035	0.031
Precipitated by alcohol (Schuchardt).....	0.025	0.021
Witte.....	0.067	0.067

*Determination of the maximal and minimal amounts of amino-acid  $\alpha$ -nitrogen capable of estimation by this method.*

The early experimental results described in this paper (page 226) had shown that although some of the amino-acids could be accurately estimated when the solution contained 0.1 mgm. of nitrogen per cc., yet other amino-acids could be estimated only when the nitrogen was only 0.05 mgm. per cc. This value is taken as the maximal value. To determine the minimal value two series of experiments were performed, one on an alanine solution, the other on an ereptone solution. A standard solution of alanine and a solution of ereptone were diluted by known amounts of water and colorimetric estimations made of the amino-acid  $\alpha$ -nitrogen present in the diluted solutions. The coloring matter produced was diluted to a suitable volume so that the colorimetric readings did not differ from the standard by large amounts. The theoretical amounts given against the results obtained for ereptone solutions were obtained by calculating from the mean result of the colorimetric and Van Slyke determinations given on page 228.

		mgm.	mgm.	mgm.	mgm.
Alanine.....	Found	0.050	0.025	0.012	0.005
Per cc.....	Calculated	0.050	0.025	0.012	0.006
Ereptone.....	Found	0.065	0.032	0.016	0.008
• Per cc.....	Calculated	0.067	0.033	0.016	0.008



It will thus be seen that the method is accurate over a range of 0.05 mgm. to 0.005 mgm. per cc. Whether amounts less than 0.005 mgm. can be estimated, we have not determined. At that concentration the amount of coloring matter produced is so small that it can only be measured with difficulty. Qualitatively, however, it is possible to detect as little as 0.001 part of a mgm. of amino-acid  $\alpha$ -nitrogen in 1 cc. of solution. This, in the case of alanine, means the detection of one part of the amino-acid in a little over 1,500,000 parts of water.

The question naturally arises as to whether the reaction will proceed quantitatively in presence of other substances. From the results obtained with ereptone (page 228) and the various peptones (page 229), the method can be used for the determination of the amino-acid  $\alpha$ -nitrogen set free in the hydrolysis of proteins. Its application to the analysis of urine and blood, however, is still attended with difficulties, ammonium salts and urea causing disturbances in the reaction. These points are being investigated, and the results will be reported shortly.

#### SUMMARY.

1. A method has been devised for the estimation of amino-acid  $\alpha$ -nitrogen by the use of triketohydrindene hydrate and pyridine.
2. The method possesses an accuracy equal to that of the Van Slyke method.
3. It will estimate within the ranges of 0.005 mgm. to 0.05 mgm. amino-acid  $\alpha$ -nitrogen per cc.
4. The method is inaccurate, however, for cystine.
5. The method is applicable to the determination of amino-acid  $\alpha$ -nitrogen (in neutral solution) set free in protein hydrolysis.

## THE METABOLISM OF VEGETARIANS AS COMPARED WITH THE METABOLISM OF NON-VEGETARIANS OF LIKE WEIGHT AND HEIGHT.

By FRANCIS G. BENEDICT AND PAUL ROTH.

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Boston, and the Battle Creek Sanitarium, Battle Creek.)

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Perhaps no special dietetic regime is more successful in attracting adherents and holding them than is that of vegetarianism. While the modern conception of the word "vegetarianism" is by no means as strictly applied to the exclusive use of vegetables as was formerly the case, and we have at the present time the lacto-vegetarians and the ovo-vegetarians, nevertheless there are a considerable number of individuals who regularly confine themselves to a strictly vegetarian diet.

Large groups of people, particularly in foreign countries, are by long custom vegetarians. In this study, however, we are specially concerned with a considerable number of individuals who, notwithstanding the fact that they live among people partaking of an ordinary mixed diet, yet adhere closely to a vegetarian diet. This adherence to a vegetarian diet may be due to any one of several causes: First, to environment, heredity, training, or the habit of the household and parents; second, to a religious belief, in which the use of flesh for food is proscribed; third, as the result of some dietetic alteration incidental to the treatment of disease and a subsequent adherence to the vegetarian diet; or finally, for physiologic or biologic reasons, in the belief that a non-flesh dietary is the natural or physiologic diet for human beings as well as other primates.

In the discussion of the supposed benefits of vegetarianism, considerable stress has been laid upon the remarkable endurance<sup>1</sup>

<sup>1</sup> I. Fisher: *Yale Med. Jour.*, xiii, p. 205, 1907. See also Ioteyko and Kipiani: *Rev. de la Soc. scient. d'hyg. alimentaire*, iii, p. 114, 1906.

apparently shown by vegetarians over flesh eaters, the statements being made that the vegetarians live upon a distinctly lower metabolic plane, are not so highly stimulated as the flesh eaters, have a lower blood pressure, and, in general, that the metabolic activities are on a lower level.

Fortunately, while it is wholly impossible to measure accurately and scientifically many of the indices of benefits or lack of benefits commonly cited by individuals, it is perfectly feasible to measure the metabolic plane by studying the respiratory exchange.<sup>2</sup> Thanks to the kindness and interest of Dr. J. H. Kellogg of the Battle Creek Sanitarium, Battle Creek, Michigan, we were enabled to make a series of observations on both men and women vegetarian subjects, all of whom may be definitely classified as normal individuals. The greater number of the subjects were members of the staff of the Sanitarium. These individuals had been meat abstainers for varying lengths of time, the periods being stated in the list given below. It is safe to state that those designated in this list as strict vegetarians probably did not eat meat of any kind more than two or three times a year.

*Men.*

- O. N. A. Strict vegetarian for 6 mos.
- B. N. C. Strict vegetarian for at least 10 yrs.
- V. E. H. Strict vegetarian for 1 yr.
- B. K. Practically a vegetarian for 2 yrs. Used meat probably once a month on the average.
- W. B. Vegetarian for 5 to 6 yrs. Has eaten meat not oftener than 2 or 3 times a yr. during that time.
- F. E. M. Strict vegetarian for 1½ yrs.
- Dr. P. R. Strict vegetarian for many yrs., and practically all his life.
- E. H. T. Strict vegetarian for 8 mos.
- E. J. W. Strict vegetarian for over 20 yrs.
- L. H. W. Except for a short period of 3 to 4 wks. (several yrs. ago) has been practically always a vegetarian. For several yrs. before the test had not eaten meat oftener than once a mo.
- T. H. Y. Strict vegetarian for over 5 yrs.

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<sup>2</sup> For a report of the most important study of the respiratory exchange of vegetarians thus far made, see W. Caspari: *Arch. f. d. ges. Physiol.*, cix, p. 473, 1905.

*Women.*

- Miss O. A. Strict vegetarian for 2 yrs.  
Mrs. E. B. Vegetarian diet for 3 mos. only.  
Miss J. U. B. Strict vegetarian for 2½ yrs.  
Miss L. B. Very seldom ate meat at any time, practically none for 10 yrs.; none for 2½ yrs. before test.  
Dr. M. D. Strict vegetarian for 20 yrs., with the use of meat at very infrequent intervals, not more than 2 or 3 times a yr.  
Miss M. H. Strict vegetarian for 2 yrs.  
Miss M. J. Strict vegetarian for 6 wks. only.  
Miss L. K. Strict vegetarian for 1 yr.; ate meat only on rare occasions.  
Mrs. A. L. Vegetarian for 5 or 6 yrs. Had eaten meat not oftener than 2 or 3 times a yr. during that time.  
Miss J. T. Strict vegetarian for 1 yr.  
Miss C. Z. Practically a life vegetarian. Had eaten no meat for 14 yrs.

The unit respiration apparatus was used for this study, all due precautions being taken as to muscular repose, absence of food in the stomach, and a non-febrile temperature; every effort was made to approximate the simplest and most perfect physiological condition. Observations were carried out with some subjects on but one or two days, but frequently they were made on several days. All recorded values were derived from not less than two satisfactorily agreeing experimental periods.

In comparing the results, it is obviously improper to obtain simply a grand average for all the experiments with vegetarians and compare this with an average for the experiments with non-vegetarians. We have, therefore, adopted the plan of comparing the vegetarians with control individuals living on a mixed diet and of the same sex and approximately the same weight and height. The ages of our subjects ranged between 20 and 40 years, and we have only occasionally to deal with the possible influence of greater age; these cases will be specially noted.

In the majority of instances it was not possible to select a normal individual comparing exactly in height and body weight with a vegetarian; and, indeed, it would be wholly erroneous to select any one individual for this comparison. We have, therefore, divided the comparisons into groups, using for each group several non-vegetarian individuals having approximately the same height and weight. Unfortunately the number of vegetarians studied, while considerably greater than ever before observed,

TABLE I.

*Comparison of the heat production of vegetarians and non-vegetarians.  
(Experiments with men.)*

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
GROUP I					
Vegetarian					
F. E. M.....	75.0	164	1698	22.7	775
Non-vegetarian					
F. A. R.....	74.4	163	1704	22.9	782
GROUP II					
Vegetarian					
E. H. T.....	64.7	170	1499	23.2	757
Non-vegetarian					
F. P. R.....	65.1	173	1543	23.7	775
D. M.....	64.0	171	1651	25.8	838
M. J. S.....	63.7	170	1647	25.9	840
GROUP III					
Vegetarian					
L. H. W.....	60.0	179	1530	25.5	810
B. K.....	58.2	178	1393	23.9	753
Non-vegetarian					
W. G. J.....	60.5	175	1746	28.9	919
L. E. E.....	59.8	175	1707	28.5	908
Dr. S.....	58.5	181	1331	22.8	716
D. J. M.....	58.0	175	1615	27.8	878
H. F. T.....	57.8	179	1348	23.3	733
GROUP IV					
Vegetarian					
T. H. Y.....	59.2	169	1605	27.2	861
Non-vegetarian					
A. L.....	60.6	171	1576	26.0	829
H. B. R.....	60.5	168	1487	24.6	783
J. B. T.....	60.1	171	1748	29.1	925
W. F. B.....	60.1	168	1632	27.2	863
E. T. W.....	57.8	169	1472	25.5	800
P. F. J.....	57.2	167	1616	28.3	883
A. G. E.....	57.0	169	1531	26.9	841
GROUP V					
Vegetarian					
W. B. L.....	59.3	164	1451	24.5	776
Non-vegetarian					
H. H. A.....	62.3	164	1487	23.9	770
S. A. R.....	60.8	165	1460	24.0	768
H. B. R.....	60.5	168	1487	24.6	783
P. F. J.....	57.2	167	1616	28.3	883

TABLE I—Concluded.

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
GROUP VI					
<i>Vegetarian</i>					
Dr. P. R.....	55.2	164	1341	24.3	753
<i>Non-vegetarian</i>					
P. F. J.....	57.2	167	1616	28.3	883
M. B.....	53.6	160	1455	27.1	831
GROUP VII					
<i>Vegetarian</i>					
O. N. A.....	55.4	171	1545	27.9	863
<i>Non-vegetarian</i>					
L. D. A.....	57.1	171	1539	27.0	844
A. G. E.....	57.0	169	1531	26.9	841
J. C. C.....	56.1	173	1522	27.1	846
C. H. H.....	55.1	169	1421	25.8	798
GROUP VIII					
<i>Vegetarian</i>					
B. N. C.....	50.6	179	1510	29.8	893
<i>Non-vegetarian</i>					
A. F. G.....	53.9	175	1453	27.0	826
L. E. A.....	52.2	174	1541	29.5	896
GROUP IX					
<i>Vegetarian</i>					
E. J. W.....	50.0	155	1158	23.2	693
<i>Non-vegetarian</i>					
I. A. F.....	54.9	156	1612	29.4	906
J. H.....	46.3	154	1223	26.4	769
GROUP X					
<i>Vegetarian</i>					
V. E. H.....	49.3	163	1365	27.7	822
<i>Non-vegetarian</i>					
J. J. G.....	50.2	164	1425	28.4	848
T. M. C.....	48.5	165	1292	26.6	788

is still too small to permit grouping, and, as a rule, only one vegetarian is used in each comparison. For this reason, we distinctly decline to draw conclusions from the individual comparisons, as they can be intelligently drawn only from the general picture.

The comparison of the male subjects is given in Table I. The subjects are here divided into ten groups, corresponding to

eleven vegetarians, as, except in the case of Group III, no two can properly be classified in a single group. In choosing individuals living on a mixed diet for comparison, the sole bases for selection have been those of body weight and body height. In other words, every attempt has been made to secure a purely objective comparison. An examination of this table shows that there is no striking uniformity in the results, the metabolism of the vegetarians being at times somewhat lower than that of the control. Occasionally the vegetarians show a higher metabolic plane than any of the control subjects. On the other hand, the vegetarians Dr. P. R., Group VI, and E. J. W.,<sup>3</sup> Group IX, show a noticeably lower metabolism than either of the controls with whom they are compared.

Believing that a comparison of the average metabolism of each group of controls with the average metabolism of the vegetarian in the same group will give a better picture, the averages have been brought together in Table II. Here for the first time we are permitted to obtain an average of all the controls with an average of all the vegetarians, since we are now comparing vegetarians with control individuals of approximately the same body weight and height. Accordingly in these final summary tables we may compare simply the heat per kgm. of body weight and per square meter of body surface.

In the averages for the ten groups, the vegetarians show a higher metabolism than the controls in four cases. In the grand average the metabolism of the vegetarians (25.5 calories per kgm.) is approximately 4 per cent lower than that of the non-vegetarians (26.4 calories per kgm.). The same small difference is found when the comparison is made on the basis of heat per square meter of body surface.

<sup>3</sup> The extraordinarily low values found with E. J. W. may in part be explained by the fact that he was the oldest man entering into this comparison, being 58 years old, and evidence has accumulated to show that age affects materially the intensity of the metabolism. Singularly enough, while the oldest woman entering into the comparison (Mrs. E.B.) is 53 years old, there is no noticeable difference in her metabolism over that of the other women vegetarians, although it is possibly of significance that she had been a vegetarian but 3 months. In both sets of comparisons the effort has been made to select controls that would be least affected by differences in age.

From a cursory examination of all the available data in this laboratory for normal individuals, it early became apparent that the metabolism of men may not properly be compared directly with that of women, and hence in this critical study of the metabolism of vegetarians we have felt it important to compare men with men and women with women. The values found for the women vegetarians and their controls are given in nine groups in Table III. In only two groups, namely, Groups I and VIII,

TABLE II.  
*Summary of comparison of vegetarians and non-vegetarians.  
(Experiments with men.)*

GROUP NO.	HEAT PRODUCTION PER 24 HRS. (COMPUTED)			
	Per kgm. of body weight		Per square meter of body surface	
	Vegetarians	Non- vegetarians	Vegetarians	Non- vegetarians
	cal.	cal.	cal.	cal.
I.....	22.7	22.9	775	782
II.....	23.2	25.1	757	818
III.....	24.7	26.3	782	831
IV.....	27.2	26.8	861	846
V.....	24.5	25.2	776	801
VI.....	24.3	27.7	753	857
VII.....	27.9	26.7	863	832
VIII.....	29.8	28.3	893	861
IX.....	23.2	27.9	693	838
X.....	27.7	27.5	822	818
Average.....	25.5	26.4	798	828

is it possible to include more than one vegetarian. An examination of the data shows that here again there is no striking uniformity in the direction of the values. Thus the two individuals, Dr. M. D. and Miss O. A. in Group I, had a measurably higher metabolism than the control, Miss H. H. Similarly, Miss C. Z. in Group II, had a considerably higher metabolism than any one of the three controls with whom she is compared. On the other hand, the vegetarians Miss J. U. B., Group V, and Miss L. B., Group VII, had a metabolism very considerably less than any one of the controls in the same group.



TABLE III.

*Comparison of the heat production of vegetarians and non-vegetarians.  
(Experiments with women.)*

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
<b>GROUP I</b>					
<i>Vegetarian</i>					
Dr. M. D.....	93.6	165	1765	18.9	695
Miss O. A.....	90.2	164	1756	19.5	708
<i>Non-vegetarian</i>					
Miss H. H.....	88.3	161	1591	18.1	652
<b>GROUP II</b>					
<i>Vegetarian</i>					
Miss C. Z.....	67.2	170	1521	22.7	747
<i>Non-vegetarian</i>					
Miss S.....	65.5	171	1426	21.8	713
Miss C. H.....	63.4	166	1413	22.3	722
Miss A. K.....	63.2	171	1402	22.2	717
<b>GROUP III</b>					
<i>Vegetarian</i>					
Mrs. E. B.....	58.0	163	1415	24.4	769
<i>Non-vegetarian</i>					
Miss B. W.....	59.4	162	1546	26.0	827
Miss J. C.....	55.1	162	1363	24.8	764
<b>GROUP IV</b>					
<i>Vegetarian</i>					
Miss L. K.....	56.8	166	1365	24.1	750
<i>Non-vegetarian</i>					
Miss M. W.....	58.6	167	1429	24.4	768
Miss M. P.....	58.1	168	1518	26.2	823
Miss G. L.....	55.0	166	1480	27.0	832
Miss M. T.....	54.5	164	1359	25.0	770
Miss F. K.....	54.1	164	1262	23.4	716
<b>GROUP V</b>					
<i>Vegetarian</i>					
Miss J. U. B.....	53.8	160	1215	22.6	694
<i>Non-vegetarian</i>					
Miss J. C.....	55.1	162	1363	24.8	764
Miss F. E.....	53.1	162	1391	26.2	799
Miss B.....	52.2	158	1415	27.2	823
Miss R. M.....	52.1	162	1353	26.0	787

TABLE III—Concluded.

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
GROUP VI					
Vegetarian					
Miss M. H.....	49.1	151	1178	24.0	712
Non-vegetarian					
Miss R. A.....	50.8	155	1293	25.5	765
Miss G. F.....	48.5	155	1233	25.4	754
GROUP VII					
Vegetarian					
Miss L. B.....	47.0	167	1168	24.9	730
Non-vegetarian					
Miss I. B.....	50.1	166	1235	24.7	737
Miss E. T.....	46.7	164	1336	28.6	838
GROUP VIII					
Vegetarian					
Mrs. A. L.....	44.9	159	1272	28.3	815
Miss M. J.....	44.8	157	1189	26.5	767
Non-vegetarian					
Miss H. T.....	45.0	159	1393	30.9	896
Miss J.....	43.0	159	1158	26.9	766
GROUP IX					
Vegetarian					
Miss J. T.....	40.0	168	1260	31.7	881
Non-vegetarian					
Miss A. C.....	42.6	165	1168	27.4	779

As noted with men, a comparison of the average values for women, irrespective of weight and height, may not properly be made; nevertheless the values for the separate groups may be averaged and thus a grand average obtained which gives a reasonably correct picture of the probable relationship between the vegetarian and non-vegetarian women. These averages, which are calculated on the basis of per kgm. of body weight and per square meter of body surface, are given in Table IV. The vegetarians show a higher metabolism than the controls in four of the nine groups. In the grand average the heat production of the vegetarians on the bases of per kgm. of body weight and per square meter of body surface is but slightly less than the

heat production of the non-vegetarians, the difference between the two classes being a little less than 2 per cent.

From these data we may conclude that the male vegetarians have a slightly less metabolism per kgm. of body weight and per square meter of body surface than have the individuals living on a mixed diet with whom they are compared. This difference is so small, however, that as a general picture no essentially striking difference is apparent between male vegetarians and non-vegetarians. Certainly there is nothing to warrant the belief

TABLE IV.  
*Summary of comparison of vegetarians and non-vegetarians.  
(Experiments with women.)*

GROUP NO.	HEAT PRODUCTION PER 24 HRS. (COMPUTED)			
	Per kgm. of body weight		Per square meter of body surface	
	Vegetarians	Non-vegetarians	Vegetarians	Non-vegetarians
	cal.	cal.	cal.	cal.
I.....	19.2	18.1	702	652
II.....	22.7	22.1	747	717
III.....	24.4	25.4	769	796
IV.....	24.1	25.2	750	782
V.....	22.6	26.1	694	793
VI.....	24.0	25.5	712	760
VII.....	24.9	26.7	730	788
VIII.....	27.4	28.9	791	831
IX.....	31.7	27.4	881	779
Average.....	24.6	25.0	753	766

that the male vegetarian subsists upon a materially lower metabolic plane. With the female vegetarians, the slight difference in metabolism shown by the male vegetarians entirely disappears. We may, therefore, fairly conclude that living upon a vegetarian diet for a longer or shorter period does not fundamentally alter the basal gaseous metabolism.

One of the particular reasons for instituting a study of the metabolism of vegetarians is to note if the body of the vegetarians contains a larger proportion of readily combustible carbohydrate

(glycogen) as the result of their special diet. Obviously vegetarians, in order to secure the total energy required for the day, must secure a larger proportion of energy from carbohydrate than do individuals living on a mixed diet; for it is much easier to obtain a larger proportion of animal fat than of vegetable fat. It was thought possible that vegetarians, subsisting for a long time on a diet rich in carbohydrates, would have acquired a storage of glycogen above that of an individual living on a mixed diet, and that consequently in a quiet, resting condition, twelve hours after the last meal, they would show a katabolism with a larger proportion of carbohydrate. This would obviously be indicated by the respiratory quotient.

In any comparison of the respiratory quotients it should be taken into consideration that this factor is extremely difficult to determine with great accuracy, since all the errors incidental to the determination of both the carbon dioxide production and the oxygen consumption affect the values for the respiratory quotient. One must, therefore, speak somewhat guardedly in comparing respiratory quotients. On the other hand, we have available respiratory quotients obtained upon twenty-two vegetarians (and here it is unnecessary, for the present at least, to make distinctions between male and female vegetarians and between individuals of different height, weight, and age). We may properly compare these values with respiratory quotients previously determined upon 132 individuals subsisting on a mixed diet. In such a comparison deductions may reasonably be drawn from an average quotient. The average respiratory quotient found with the 22 vegetarians (*i.e.*, 11 men and 11 women), was 0.83, while the average quotient found with 132 individuals subsisting on a mixed diet (77 men and 55 women) was 0.81. This difference is slight and is wholly incompatible with the belief that vegetarians, when in the post-absorptive condition, have available any considerably larger proportion of easily combustible carbohydrate material than have non-vegetarians.



## THE METABOLISM OF ATHLETES AS COMPARED WITH NORMAL INDIVIDUALS OF SIMILAR HEIGHT AND WEIGHT.

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The special influence of athletic training upon the basal resting metabolism, particularly of the fully trained athletic condition, has received scant attention in studies of normal metabolism. It is true that in the researches carried out by the Zuntz school, especially those in which the work of marching was studied, the influence of training upon the muscular efficiency for external work was considered; furthermore, certain writers have recognized the desirability of indicating the general muscular development of their subjects, and we find occasional comments in the literature upon the relationship between muscular condition and the basal metabolism.

Thus Speck<sup>1</sup> states that muscular individuals consume more oxygen and produce more carbon dioxide than do non-athletic persons under similar conditions. In the various summaries of measurements of the basal metabolism, Magnus-Levy and Falk,<sup>2</sup> and Loewy<sup>3</sup> have noted the muscular condition of their subjects. Magnus-Levy and Falk<sup>4</sup> conclude that three individuals with special muscular development showed a basal metabolism which was no greater and possibly somewhat smaller than other

<sup>1</sup> C. Speck: *Physiologie des menschlichen Athmens*, Leipsic, 1892, p. 224.

<sup>2</sup> A. Magnus-Levy and E. Falk: *Arch. f. Anat. u. Physiol.*, Supplement, p. 321, 1899.

<sup>3</sup> A. Loewy: *Oppenheimers Handbuch der Biochemie*, Jena, iv, p. 179, 1911.

<sup>4</sup> Magnus-Levy and Falk: *loc. cit.*, p. 363.

individuals; per unit of body weight they showed a somewhat smaller metabolism than the average individual.

In a study made of the metabolism of the vegetarian athlete, Karl Mann, Caspari<sup>5</sup> concluded from the higher basal metabolism that this individual had a much more powerful musculature than another subject of essentially the same weight. Mann had a height of 163.5 cm. and a body weight of 61.175 kgm. Herr B., with whom he was compared, was 175 cm. in height and weighed 63.18 kgm.

Benedict and Carpenter,<sup>6</sup> in reporting a number of experiments on the metabolism of normal individuals, included incidentally certain comparisons of four athletes with a number of non-athletes having approximately the same body weight and height. No special effort was made to study the metabolism of athletes in particular, save in the observations with a professional bicycle rider. The other three subjects were college athletes, one of whom was a trained bicycle rider, another a football player, and the third especially proficient in gymnasium work. All four athletes showed a distinctly lower metabolism, both per kgm. of body weight and per square meter of body surface, than did the normal individuals with whom they were compared. As was pointed out by the writers, these experiments were not planned primarily with the idea of comparing the athletes with a group of non-athletes. Neither were the experiments made under ideal conditions for comparison; for it has been clearly shown that the best method of determining the basal metabolism of different individuals is with the subject in the post-absorptive state and with complete muscular rest. These individuals were compared on an entirely different basis, as they were not invariably in the post-absorptive state and were all sitting more or less quietly in the calorimeter chamber. Nevertheless the fact that the results were so uniformly low with the four subjects cannot be overlooked, and, as the authors themselves stated, a special research on this question was needed before an intelligent comparison of the basal metabolism of athletes and non-athletes could be made.

<sup>5</sup> W. Caspari: *Arch. f. d. ges. Physiol.*, cix, p. 473, 1905.

<sup>6</sup> F. G. Benedict and T. M. Carpenter: *Carnegie Institution of Washington Publications*, No. 126, p. 236, 1910.

As the experimental data for normal values accumulated in the Nutrition Laboratory, it became evident that the body composition probably had a considerable influence upon the metabolism. When all the results were superficially inspected, the general picture appeared to be that distinctly fat individuals showed a low metabolism per kgm. of body weight and per square meter of body surface. It seemed therefore of double interest to study the effect upon the basal metabolism of a greater than normal proportion of protoplasmic material such as was present in the body of the trained athlete.

No opportunity presented itself for the study of a group of trained athletes until the winter of 1912-1913, when one of us made a series of observations in the chemical laboratory of Syracuse University. The subjects of this study were selected from among the students of the University; during the experiments they continued to eat at their respective college clubs, confining themselves entirely to the plainer articles of diet. Practically none of them were using tobacco, although it is possible that some occasionally indulged.

The sports engaged in were crew practice, 16 pound hammer throw and shot put, one-quarter, one-half, and one mile runs, 100 yard dash, relay team, and basket ball; during the previous year several of the men had played football. While they were taking part in their respective sports daily, they were not in that degree of training which might be expected of a professional prize fighter or a 6 day bicycle rider. Neither were they all of an equal degree of proficiency; a few were in their first season of regular sports, but others had been competing not only during their entire college course but had competed in interscholastic meets before entering college. One of the men a few weeks later was the first winner in an event at the Olympiad in Stockholm, another in an intercollegiate meet at Philadelphia, and others won in their events in the interclass meets. Broadly speaking, they were all active young men, participating regularly from one to three hours a day in sports.

The results obtained with these athletes are compared in Table I with observations made upon non-athletes of similar height and weight. With the values obtained with the college athletes other values are included which were secured with the professional



TABLE I.

*Comparison of the heat production of athletes and normal non-athletic men.*

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
<b>GROUP I</b>					
<i>Athlete</i>					
W. S. ....	88.5	165	2017	22.8	823
<i>Non-athlete</i>					
O. F. M. ....	85.8	171	1827	21.3	761
<b>GROUP II</b>					
<i>Athletes</i>					
J. H. R. ....	82.2	187	1978	24.1	849
D. H. W. ....	82.1	186	2034	24.8	873
M. H. K. ....	79.0	188	1944	24.6	856
E. G. ....	78.9	184	2126	27.0	940
<i>Non-athletes</i>					
F. G. B. ....	83.1	183	1802	21.7	770
W. A. M. ....	78.0	183	1816	23.3	807
<b>GROUP III</b>					
<i>Athlete</i>					
F. G. R. ....	74.0	179	1914	25.9	882
<i>Non-athletes</i>					
W. J. T. ....	74.2	183	1770	23.9	816
C. B. S. ....	71.1	179	1700	23.9	806
<b>GROUP IV</b>					
<i>Athletes</i>					
C. D. R. ....	74.0	173	1908	25.8	879
H. R. W. ....	73.9	175	1842	24.9	848
<i>Non-athletes</i>					
Dr. M. ....	75.9	175	1877	24.7	849
J. P. C. ....	73.7	169	1526	20.7	706
H. W. E. ....	73.0	168	1559	21.4	725
<b>GROUP V</b>					
<i>Athlete</i>					
P. D. F. ....	71.2	176	1810	25.4	858
<i>Non-athletes</i>					
C. B. S. ....	71.1	179	1700	23.9	806
J. H. H. ....	69.1	171	1634	23.6	789
B. A. W. ....	67.9	174	1945	28.6	949
<b>GROUP VI</b>					
<i>Athlete</i>					
M. A. M. ....	66.0	176	1695	25.7	843

TABLE I—Concluded.

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
<i>Non-athletes</i>					
B. A. W.....	67.9	174	1945	28.6	949
F. P. R.....	65.1	173	1543	23.7	775
J. J. C.....	65.0	175	1585	24.4	796
R. G.....	62.7	173	1590	25.4	820
GROUP VII					
<i>Athletes</i>					
M. Y. B.....	63.5	172	1677	26.4	856
R. D. S.....	63.5	170	1619	25.5	826
<i>Non-athletes</i>					
F. P. R.....	65.1	173	1543	23.7	775
J. J. C.....	65.0	175	1585	24.4	796
E. H. T.....	64.7	170	1499	23.2	757
D. M.....	64.0	171	1651	25.8	838
M. J. S.....	63.7	170	1647	25.9	840
R. G.....	62.7	173	1590	25.4	820
H. C. B.....	62.0	173	1653	26.7	856
GROUP VIII					
<i>Athlete</i>					
W. F. M.....	62.4	180	1816	29.1	936
<i>Non-athletes</i>					
K. H. A.....	66.4	182	1654	24.9	819
J. R.....	66.0	182	1679	25.4	835
Dr. S.....	58.5	181	1331	22.8	716
GROUP IX					
<i>Athlete</i>					
C. J. D.....	56.7	160	1524	26.9	838
<i>Non-athletes</i>					
Dr. P. R.....	55.2	164	1341	24.3	753
I. A. F.....	54.9	156	1612	29.4	906
M. B.....	53.6	160	1455	27.1	831
GROUP X					
<i>Athlete</i>					
W. A. S.....	56.3	169	1562	27.7	863
<i>Non-athletes</i>					
E. T. W.....	57.8	169	1472	25.5	800
P. F. J.....	57.2	167	1616	28.3	883
L. D. A.....	57.1	171	1539	27.0	844
A. G. E.....	57.0	169	1531	26.9	841
C. H. H.....	55.1	169	1421	25.8	798

bicycle rider, M. A. M., who was employed as a subject by Dr. E. P. Cathcart<sup>7</sup> at the Nutrition Laboratory in the winter of 1911-1912.

In making such a comparison several difficulties are immediately encountered. In the first place the athletes were not all of the same height and weight; hence it is necessary to compare them with the non-athletes individually and not collectively. Furthermore, when an individual of a given weight and height is to be compared with other individuals of the same weight and height, it is obvious that unless a very large number of control individuals are available to select from it is extremely difficult to obtain adequate normal data. A certain amount of difference, both in weight and height, must therefore be allowed in the collection of a suitable number of individuals for comparison. We have attempted to select the values impartially, making our choice of controls on the basis of weight and height as nearly as possible identical with those of the athletes. The values for all these comparisons are taken from the large table given in the paper on the basal gaseous metabolism of men and women which was recently published by us in conjunction with Emmes and Roth.<sup>8</sup> Further details regarding these subjects may be obtained by referring to the original publication.<sup>9</sup>

In the comparison of the metabolism of athletes and non-athletes the values are divided into ten groups (see Table I), the arrangement being in the order of the decreasing body weight. The initials, body weight, and height of each subject are given. Since individuals of approximately the same height and weight are compared, the total heat production, calculated for twenty-four hours, is recorded. It is thus seen that the comparison between the two classes in each group may properly be made upon the basis of total heat production alone. On the other hand, since it is the habit of many writers to consider in such comparisons the heat production per kgm. of body weight per twenty-four

<sup>7</sup> F. G. Benedict and E. P. Cathcart: *ibid.*, No. 187, 1913.

<sup>8</sup> F. G. Benedict, L. E. Emmes, P. Roth, and H. M. Smith: *this Journal*, xviii, p. 139, 1914.

<sup>9</sup> Two corrections, which slightly affect the values in the original table, are a decrease of 5 cm. in the height of Dr. M. and an increase of 3 kgm. in the weight of Dr. R.

hours, and particularly the heat production per square meter of body surface as computed by the Meeh formula per twenty-four hours, these are likewise included in the table.

An examination of Table I shows that the first eight athletes, *i.e.*, those in Groups I, II, III, and IV, had with but one exception a greater total heat production per twenty-four hours on the three bases of comparison than any of the non-athletic individuals with whom they are compared. These differences are for the most part of considerable magnitude, being from 200 to 300 calories. The professional athlete, M. A. M., in Group VI shows a considerably higher heat production per twenty-four hours than do three of the four non-athletes with whom he is compared. The fourth non-athlete, B. A. W., had a very high metabolism of 1945 calories per twenty-four hours, 28.6 calories per kgm. of body weight per twenty-four hours, and 949 calories per square meter of body surface per twenty-four hours. This subject, although classed as a non-athlete, was an assistant machinist in the Nutrition Laboratory, and accustomed to doing comparatively heavy machine work, piping, and boiler work. Although a well developed and somewhat muscular individual, he could not possibly be classified as a trained, hardened athlete. He is retained in this comparison to show the possibilities of an increased metabolism in individuals without a distinctly athletic training. It should be stated, however, that this is by far the most extraordinary case we have ever noted of so great an intensity of metabolism in an individual of this height and weight.

The general picture presented in Group VII, in which M. Y. B. and R. D. S. are compared with seven non-athletes, shows no difference in favor of either class. On the other hand, with the athletic subject W. F. M. in Group VIII there was a very large increase in the metabolism over that of the three non-athletes in the same group; in fact, this athlete had the highest metabolism per kgm. of body weight per twenty-four hours of any athlete studied.

In Group X, in which the one athlete had the smallest body weight of any of the athletes studied, there was practically no difference in the metabolism of the two classes.

It is clear from this comparison, therefore, that the heaviest athletes, with a body weight of 65 kgm. or over, had almost

invariably a pronounced increase in the metabolism above that of the non-athletic individuals of approximately the same body weight and height with whom they are compared. On the other hand, with athletes below 65 kgm., with the single exception of W. F. M., the difference is by no means so striking. It is evident that further observations with athletes of this weight are much needed to complete the study. Nevertheless, the evidence is sufficiently plain to show that the observations of Benedict and Carpenter were distinctly deficient, since their four subjects all showed a considerably lower metabolism per kgm. of body weight and per square meter of body surface than did the normal individuals. In this study, however, we find that none of the athletes show a characteristically lower metabolism than the non-athletes, and that a very large proportion of them show a considerable increase in the metabolism.

While the method of presentation outlined has been used, as no other method is available, we expressly wish to deny the legality of basing conclusions upon any individual comparisons or, indeed, the comparisons in any given groups. In Table II, therefore, we give the average value for each group and also a grand average for all of the athletes and non-athletes. Owing to the unequal number of athletes and non-athletes, it is somewhat inconsistent to find the grand averages and draw deductions from these values. On the other hand, an inspection of the general picture as shown in Table II is perfectly legitimate and this must be the only determining factor in drawing final conclusions.

Of the ten groups which are averaged in Table II, we find that the heat per kgm. of body weight is greatest with athletes in all but two instances, *i.e.*, in Groups V and IX, in which there was no difference. In none of the comparisons did a non-athletic group have a higher metabolism than the athletes with whom they were compared. The final average also shows a higher metabolism for the athletes of 26.0 calories per kgm. of body weight per twenty-four hours as compared with 24.4 calories per kgm. for the non-athletes. On the basis of per square meter of body surface we again find that the athletes exceed the metabolism of the non-athletes in all the groups, although the increases in Groups V, VI, and IX are insignificant. The general average on this basis shows a metabolism of 863 calories per square meter of body sur-

face per twenty-four hours for the athletes as against 807 calories for the non-athletes. As has already been stated, the general averages must be taken with some reserve since an unequal number of subjects are compared in the two classes. From the general picture of the comparison, however, the conclusion may be fairly drawn that athletes have a somewhat higher metabolism, both per kgm. of body weight and per square meter of body surface, than do the non-athletes with whom we have compared them.

TABLE II.  
*Summary of comparison of athletes and non-athletes.*

GROUP NO.	HEAT PRODUCTION PER 24 HRS. (COMPUTED)			
	Per kgm. of body weight		Per square meter of body surface	
	Athletes	Non-athletes	Athletes	Non-athletes
	cal.	cal.	cal.	cal.
I.....	22.8	21.3	823	761
II.....	25.1	22.5	880	789
III.....	25.9	23.9	882	811
IV.....	25.4	22.3	864	760
V.....	25.4	25.4	858	848
VI.....	25.7	25.5	843	835
VII.....	26.0	25.0	841	812
VIII.....	29.1	24.4	936	790
IX.....	26.9	26.9	838	830
X.....	27.7	26.7	863	833
Average.....	26.0	24.4	863	807

The possible objection may be raised to these observations that although the athletes were studied in the morning, twelve hours after the last meal, yet they had been engaged in active muscular exercise the day before, and there may have been a prolonged after-effect of work. Benedict and Cathcart<sup>10</sup> have indeed shown a considerable after-effect of work, lasting for several hours after the completion of severe bicycle riding, but it is hardly to be supposed that the after-effects of work carried out the day before would so persist for over twelve hours as to account for the larger metabolism noted with the majority of these athletes. We believe we are therefore justified in concluding that the greatly

<sup>10</sup> Benedict and Cathcart: *loc. cit.*, p. 163.

increased proportion of active protoplasmic tissue present in the trained, hardened athlete is alone sufficient to account for the increase in the metabolism, and that this is not only an absolute increase, but from the nature of the comparison the metabolism is likewise increased per kgm. of body weight and per square meter of body surface. It would thus appear that the increase in the metabolism noted with athletes points strongly towards the earlier conception that the katabolism of the body is proportional not to the surface of the body, but to the active mass of protoplasmic tissue.

## A COMPARISON OF THE BASAL METABOLISM OF NORMAL MEN AND WOMEN.

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As early as 1843 we find observations made by Scharling<sup>1</sup> in which both sexes were studied as to their metabolism and respiratory exchange. The values found by Scharling and recalculated by Sondén and Tigerstedt<sup>2</sup> on the basis of carbon dioxide per hour in grams show that with a girl nineteen years of age there was a considerably less carbon dioxide excretion per hour than with either of two men or a sixteen year old boy. Scharling concludes from this single observation that there is a greater production of carbon dioxide with men than with women of the same age.

The investigation of Andral and Gavarret<sup>3</sup> was very much more extended, inasmuch as thirty-seven men and twenty-two women were studied. Andral and Gavarret conclude that throughout the whole of life there is a greater production of carbon dioxide by men than by women, but that there is a proportionally larger production between the ages of sixteen and forty, when men, as a rule, produce about twice as much carbon dioxide as do women. Unfortunately Andral and Gavarret do not give the body weight of their subjects, and we are thus unable to compare them on the popular bases of per kgm. of body weight and per square meter of body surface.

In their classical monograph on the respiratory exchange and total metabolism of men, Sondén and Tigerstedt<sup>4</sup> published an

<sup>1</sup> E. A. Scharling: *Ann. d. Chem. u. Pharm.*, xlv, p. 214, 1843.

<sup>2</sup> K. Sondén and R. Tigerstedt: *Skand. Arch. f. Physiol.*, vi, p. 54, 1895.

<sup>3</sup> G. Andral and J. Gavarret: *Ann. de Chim. et de Phys.*, Series 3, viii, p. 129, 1843.

<sup>4</sup> Sondén and Tigerstedt: *loc. cit.*, p. 58.



extensive series of observations on both men and women in which the large respiration chamber in Stockholm was used. These results are comparable, although the observations were made under such conditions as to exclude them for use as indices of basal metabolism. Computing the values both for body weight and body surface, they conclude that in youth the carbon dioxide production of boys is considerably greater than that of girls of about the same age and the same body weight, but with increasing age this difference gradually becomes less and less and finally in old age it disappears entirely. It must be noted here that the authors specifically state that it appears to them that new experiments are necessary before this problem can be completely solved.

In 1899 Magnus-Levy and Falk<sup>5</sup> published an extended series of observations on both men and women in which the Zuntz-Geppert respiration apparatus was employed. Although Johansson<sup>6</sup> had shortly before emphasized the importance of controlling muscular repose and had outlined his experience in the voluntary exclusion of muscular activity, these observations of Magnus-Levy and Falk represent the first comparative observations made upon both men and women in which particular attention was given to complete muscular rest; hence they are more perfectly comparable with our experiments than any series published previous to 1899. The series with males comprise observations on sixteen boys, ten men between twenty-two and fifty-six years of age, and five men sixty-four years old and over. The series with women include observations on nine girls, fifteen women between seventeen and fifty-seven, and seven women seventy-one years or older. The data as to the age, weight, and height are most carefully recorded and general comments made regarding the body condition, respiration rate, and pulse rate. The authors have likewise computed the values per kgm. per minute and per square meter of body surface per minute. In their comparisons of the values obtained with men and women on the basis of per kgm. of body weight, they conclude that in middle life the gaseous metabolism of women is approximately the same

<sup>5</sup> A. Magnus-Levy and E. Falk: *Arch. f. Anat. u. Physiol., Physiol. Abt.*, Supplement, p. 314, 1899.

<sup>6</sup> J. E. Johansson: *Skand. Arch. f. Physiol.*, viii, p. 85, 1898.

as that of men of the same age and body weight. With children and old men and women, the females have a slightly less (5 per cent) metabolism than the males. The authors also point out that, owing to the larger proportion of body fat, females would have a metabolism per unit of active protoplasmic tissue greater than would men.

Although since the experiments of Magnus-Levy and Falk, a large number of observations have been made on the metabolism of men, there have been relatively few observations on normal women. These include those reported by Johansson,<sup>7</sup> which were made with unusual care as to absolute muscular repose, and a few desultory studies by members of the Zuntz school. The experiments of Benedict and Carpenter<sup>8</sup> with two women in the respiration apparatus in Middletown, Connecticut, were not made with complete muscular rest; a considerable number of observations upon non-athletic men were secured, however, under similar conditions of muscular activity, and the results were therefore comparable with those obtained with the women subjects. Both women showed a remarkably lower metabolism per kgm. of body weight and per square meter of body surface than did the men with whom they were compared.

It is thus seen that in the last sixteen years no extensive comparative study of the metabolism of normal men and women has appeared, and the last two great studies, namely, those of Sondén and Tigerstedt and of Magnus-Levy and Falk, are distinctly at variance with each other as to the final conclusions.

In accumulating normal material for comparison with pathological data in this laboratory and elsewhere, results obtained with eighty-nine men and sixty-eight women have been gathered together and presented in two tables in a paper recently published.<sup>9</sup> It is our purpose here to discuss the values included in these tables and, as far as possible, to compare the metabolism of men and women.

<sup>7</sup> Johansson: *ibid.*, xxi, p. 1, 1909.

<sup>8</sup> F. G. Benedict and T. M. Carpenter: *Carnegie Institution of Washington Publications*, No. 126, p. 238, 1910.

<sup>9</sup> F. G. Benedict, L. E. Emmes, P. Roth, and H. M. Smith: *this Journal*, xviii, p. 139, 1914.

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From the foregoing paper by Benedict and Smith,<sup>10</sup> it is obvious that the normal men in this table who are distinctly classified as athletes cannot properly be used for comparison with women, and hence a grand average of the results for men cannot properly be compared with an average of the results for women. Since, however, such a comparison is not without interest, we give in Table I an average of all of the values found with the eighty-nine men and the sixty-eight women. Perhaps the most striking feature of this comparison is the fact that although the total metabolism, as measured by the carbon dioxide production and oxygen absorption, is greater with men than with women, the body weight is also considerably greater and likewise the height.

TABLE I.  
*Average values for metabolism of normal men and women.*

NO. AND SEX OF SUBJECTS	AGE	OBSER- VATIONS		BODY WEIGHT WITHOUT CLOTHING	HEIGHT	TOTAL CARBON DIOXIDE PER MIN.	TOTAL OXYGEN PER MIN.	CARBON DIOXIDE PER KGM. PER MIN.	OXYGEN PER KGM. PER MIN.	PULSE RATE	HEAT PER 24 HRS. (COMPUTED)		
		Dys.	Periods								Total	Per kgm. of body weight	Per square meter of body surface
	yrs.		kgm.	cm.	cc.	cc.	cc.	cc.			cal.	cal.	cal.
89 men.....	26	8	28	64.3	172	196	235	3.04	3.65	61	1638	25.5	832
68 women....	27	2	5	54.5	162	158	195	2.90	3.58	69	1355	24.9	772

But when we compare the average carbon dioxide production and oxygen consumption per kgm. per minute of the men with that of the women, there is a striking agreement in the results. Thus, 3.04 cc. of carbon dioxide per kgm. of body weight were produced per minute by the men against 2.90 cc. for the women, and 3.65 cc. of oxygen were consumed by the men as compared with 3.58 cc. of oxygen by the women. In comparing the calculated heat production per twenty-four hours, the greater total amount for the men is obviously due to the larger body weight; but on the basis of per kgm. of body weight, 25.5 calories were produced with the men as compared with 24.9 calories with the women; per square meter of body surface the values are 832 calories for the men and 772 calories for the women.

<sup>10</sup> Benedict and Smith: this *Journal*, xx, p. 243, 1915.

A comparison of this nature is, however, distinctly erroneous, and while one might assume that with eighty-nine men and sixty-eight women the average figure might hold, nevertheless a careful analysis of the situation will show that this method of comparison is not wholly justifiable. Indeed, its use has been carefully avoided and rightly so by all previous writers. On the other hand, believing that only individuals of like weight and height can properly be compared, it is possible with such a large accumulation of material as is presented in the two tables previously published to compare the metabolism of women with that of men of approximately the same weight and height. Such a comparison is made in Table II. Furthermore, although disinclined to believe in the value of a comparison on the basis of heat production per kgm. and per square meter of body surface, we have included these comparisons in the table, since they have been used extensively by other writers.

The attempt in this comparison has been to secure a fair average picture of the metabolism of men and women, and to select the data for the various groups of men and women compared so as to keep the difference in height within 1 or 2 cm. and the difference in weight within 1 or 2 kgm. Unfortunately, in all instances this method of comparison cannot be adhered to, and certain deviations are absolutely necessary. While for the most part it has been possible to employ three or more men and women in each group, with certain groups, particularly Groups V, VI, VII, X, and XI, it has been impracticable to use more than two individuals. This is admittedly a failing in the method of comparison.

In both Groups I and II all the women had a lower metabolism per kgm. of body weight and per square meter of body surface than any of the men with whom they are compared. In these two groups, therefore, the evidence is strongly in favor of the general supposition that the metabolism of women is less, not only absolutely, but per kgm. and per square meter, than that of men of similar weight and height. In the subsequent groups the differences are not so noticeable until we reach Group V with unfortunately only one woman for comparison. In Group VII but one man is compared, but his metabolism is distinctly higher than that found with normal men of approximately his

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TABLE II.

*Comparison of the heat production of normal men and women of like body weight and height.*

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
<b>GROUP I</b>					
<i>Men</i>					
F. P. R.....	65.1	173	1543	23.7	775
E. H. T.....	64.7	170	1499	23.2	757
D. M.....	64.0	171	1651	25.8	838
M. J. S.....	63.7	170	1647	25.9	840
R. G.....	62.7	173	1590	25.4	820
H. C. B.....	62.0	173	1653	26.7	856
<i>Women</i>					
Miss C. Z.....	67.2	170	1521	22.7	747
Miss S.....	65.5	171	1426	21.8	713
Miss A. K.....	63.2	171	1402	22.2	717
<b>GROUP II</b>					
<i>Men</i>					
E. H. T.....	64.7	170	1499	23.2	757
M. J. S.....	63.7	170	1647	25.9	840
S. A. R.....	60.8	165	1460	24.0	768
H. B. R.....	60.5	168	1487	24.6	783
W. F. B.....	60.1	168	1632	27.2	863
T. H. Y.....	59.2	169	1605	27.2	861
<i>Women</i>					
Miss C. H.....	63.4	166	1413	22.3	722
Miss V. A.....	62.9	168	1330	21.2	682
Miss C.....	61.9	168	1427	23.1	739
<b>GROUP III</b>					
<i>Men</i>					
H. H. A.....	62.3	164	1487	23.9	770
S. A. R.....	60.8	165	1460	24.0	768
W. B. L.....	59.3	164	1451	24.5	776
<i>Women</i>					
Dr. A. B.....	60.3	163	1486	24.6	786
Miss B. W.....	59.4	162	1546	26.0	827
Mrs. E. B.....	58.0	163	1415	24.4	769
Miss M. M.....	57.9	164	1475	25.4	801
<b>GROUP IV</b>					
<i>Men</i>					
A. L.....	60.6	171	1576	26.0	829
H. B. R.....	60.5	168	1487	24.6	783
J. B. T.....	60.1	171	1748	29.1	925
W. F. B.....	60.1	168	1632	27.2	863

TABLE II—Continued.

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
GROUP IV—Continued					
<i>Men</i>					
T. H. Y.....	59.2	169	1605	27.2	861
E. T. W.....	57.8	169	1472	25.5	800
P. F. J.....	57.2	167	1616	28.3	883
L. D. A.....	57.1	171	1539	27.0	844
A. G. E.....	57.0	169	1531	26.9	841
<i>Women</i>					
Miss L. U.....	59.3	169	1448	24.4	774
Miss M. W.....	58.6	167	1429	24.4	768
Miss M. P.....	58.1	168	1518	26.2	823
GROUP V					
<i>Men</i>					
L. E. E.....	59.8	175	1707	28.5	908
D. J. M.....	58.0	175	1615	27.8	878
J. C. C.....	56.1	173	1522	27.1	846
<i>Women</i>					
Miss E. P.....	57.7	175	1430	24.9	780
GROUP VI					
<i>Men</i>					
P. F. J.....	57.2	167	1616	28.3	883
Dr. P. R.....	55.2	164	1341	24.3	753
<i>Women</i>					
Miss L. K.....	56.8	166	1365	24.1	750
Miss G. L.....	55.0	166	1480	27.0	832
GROUP VII					
<i>Men</i>					
I. A. F.....	54.9	156	1612	29.4	906
<i>Women</i>					
Mrs. D. C.....	54.9	153	1276	23.3	719
Miss L. T.....	53.6	155	1247	23.3	713
Miss B.....	52.2	158	1415	27.2	823
GROUP VIII					
<i>Men</i>					
Dr. P. R.....	55.2	164	1341	24.3	753
M. B.....	53.6	160	1455	27.1	831
<i>Women</i>					
Miss J. C.....	55.1	162	1363	24.8	764
Miss M. T.....	54.5	164	1359	25.0	770
Miss F. K.....	54.1	164	1262	23.4	716
Miss F. E.....	53.1	162	1391	26.2	799

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TABLE II—Concluded.

GROUP AND SUBJECT	NUDE WEIGHT	HEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)		
			Total	Per kgm.	Per square meter
	kgm.	cm.	cal.	cal.	cal.
<b>GROUP IX</b>					
<i>Men</i>					
J. J. G.....	50.2	164	1425	28.4	848
V. E. H.....	49.3	163	1365	27.7	822
T. M. C.....	48.5	165	1292	26.6	788
<i>Women</i>					
Miss J. B.....	51.1	163	1265	24.8	746
Miss E. C.....	50.5	164	1327	26.3	788
Miss I. B.....	50.1	166	1235	24.7	737
Miss C. B.....	49.8	162	1419	28.6	850
<b>GROUP X</b>					
<i>Men</i>					
T. M. C.....	48.5	165	1292	26.6	788
<i>Women</i>					
Miss L. B.....	47.0	167	1168	24.9	730
Miss E. T.....	46.7	164	1336	28.6	838
<b>GROUP XI</b>					
<i>Men</i>					
J. H.....	46.3	154	1223	26.4	769
<i>Women</i>					
Miss R. W.....	45.0	153	1273	28.3	816
Miss M. J.....	44.8	157	1189	26.5	767

height and weight. In Group IX the general picture would imply a metabolism somewhat higher with the men than with the women.

It is obvious that the best comparison for all these groups can only be made with the average figures; we have therefore summarized the comparisons for the several groups in Table III. From the conditions of the grouping it is obviously unnecessary to consider the total metabolism, and our comparisons may simply be confined to the heat production per kgm. and per square meter of body surface. In these eleven groups the men show a greater metabolism per kgm. of body weight than that of the women in eight cases; the average of the data shows that the men had a heat production of 26.5 calories per kgm. per twenty-four hours as compared with a heat production of 25.0 calories per kgm. for the women, or approximately a 5 per cent increase. Further-

more, on the basis of per square meter of body surface, we find a greater heat production with men in nine cases, the average being 819 calories per square meter for the men as compared with 770 calories for the women, an increment of approximately 6 per cent.

Some of the defects of this method of comparison are obvious when we consider the special groups. Thus, in Group I the men are of approximately normal body weight while the women with

TABLE III.

*Summary of comparison of normal men and women of like body weight and height.*

GROUP NO.	HEAT PRODUCTION PER 24 HRS. (COMPUTED)			
	Per kgm. of body weight		Per square meter of body surface	
	Men	Women	Men	Women
	cal.	cal.	cal.	cal.
I.....	25.1	22.2	814	726
II.....	25.4	22.2	812	714
III.....	24.1	25.1	771	796
IV.....	26.9	25.0	848	788
V.....	27.8	24.9	877	780
VI.....	26.3	25.6	818	791
VII.....	29.4	24.6	906	752
VIII.....	25.7	24.9	792	762
IX.....	27.6	26.1	819	780
X.....	26.6	26.8	788	784
XI.....	26.4	27.4	769	792
Average.....	26.5	25.0	819	770

whom they are compared are larger than normal, and in this group we find a greater disproportion between men and women than in any of the others. On the other hand, in Groups X and XI, in which small individuals are compared, the metabolism is slightly higher with women than with men; if we are to explain this on the basis of an actual difference in the active mass of protoplasmic tissue, we must infer that with small, thin women the proportion of subcutaneous fat is no greater than that with small, thin men.

It would appear from these comparisons, therefore, that the



metabolism of men is about 5 or 6 per cent greater than that of women with like height and body weight. Since athletes have been carefully excluded in this selection of material, we deal here only with approximately normal individuals, and in any event individuals of similar size and form appear in the comparative groups. The slight increment in the metabolism of men over women may be explained on several grounds. First, there may be a disproportion between body weight and body surface; in other words, the Meeh formula may not hold with women as it does with men. Doubtless in individual cases this may play a part; but with as large a number of individuals as are here studied, we think it fair to assume that this disproportion is more theoretical than actual. Secondly, there is in all probability with women, particularly in the groups with the greater body weight, a larger proportion of subcutaneous fat than with men, and conversely with men a larger proportion of active protoplasmic tissue. Finally, it should be stated that while the age of certain individuals in the comparison should properly be considered, the greater number of our experiments were made with individuals between twenty and thirty years of age, and hence no individual case can in any way affect the general deduction; *i.e.*, that men have a slightly greater metabolism per kgm. of body weight and per square meter of body surface than have women of like weight and height.

## FACTORS AFFECTING BASAL METABOLISM.

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Superficial observations have led us to the general belief that the vital processes are much the same with average individuals. It is true that we are inclined to classify people as high-strung and nervous on the one hand and phlegmatic on the other; yet, on the average, the normal individual apparently has a metabolic plane comparable to his neighbor's, for the general uniformity in the energy requirement, as indicated by dietary studies of people of various classes performing like amounts of muscular work, suggests that there is likewise a uniformity in the basal metabolism of these individuals, *i.e.*, the metabolism during complete muscular rest and in the post-absorptive condition. Undoubtedly a group of miners will have about the same average metabolism as another group of miners, or a group of stenographers in one city will have much the same energy requirement as a group of stenographers in another city. But while the uniformity in energy requirement is of great significance in dietary studies and in the arrangement of dietaries, from the standpoint of pure physiology and for the application of physiological values to a study of pathological cases, it is of much more importance for us to know how constant is the basal metabolism with different individuals. For instance, while two groups of stenographers would have on the average the same energy requirement for the like amount of work, would the individual members of the group have the same basal metabolism?

The general impression that individuals of like occupation have similar dietary needs applies only to persons of approximately similar age and weight; for it is a matter of household observation that the growing, active child apparently requires

a much larger proportion of food than does the more sedate adult. Furthermore, it is commonly supposed that a large man would have a much greater basal metabolism than would a small man. Hence the factor of size should be considered, and it is clear that only individuals of like size should be compared. Heretofore the chief factor in determining the size of individuals has been the weight, and many physiologists, as a closer approximation to a basis for scientific comparison, have been wont to express values on the basis of the metabolism per kilogram of body weight.

Other physiologists use another index: that of the metabolism per square meter of body surface. They base their hypothesis upon one of the two current explanations of heat production in the body: first, that heat is incidental to the vital processes, all the energy used by the body being finally degraded to heat which, as the end-product, is lost from the body; and, second, that heat is produced primarily to keep the body warm.

Of these two views, the basal idea in the second theory has obtained for many years. It is a well known fact that in health the normal body temperature of approximately 37°C. remains relatively constant throughout life. The body is continually losing heat; since the body must be kept at a normal temperature, heat must be produced to supply the heat lost. The production of this heat may take place in two ways: first, by active external muscular work, such as would be exemplified by teamsters swinging their arms in cold weather to keep warm; second, by internal muscular activity, such as increased blood flow, muscular tonus, etc.

The development of the belief that the heat is produced primarily to keep the body warm may easily be traced from the old conception of the animal organism as a cooling body to which Newton's law of cooling applies. As early as 1843, Bergmann<sup>1</sup> promulgated this idea, although he gave no experimental evidence. Forty years later Rubner,<sup>2</sup> in his classical research on

<sup>1</sup> C. Bergmann and R. Leuckart: *Anatomisch-physiologisches Übersicht des Thierreichs*, Stuttgart, 1852, p. 272. See also Bergmann: *Wärmeökonomie der Thiere*, Göttingen, 1848, p. 9.

<sup>2</sup> M. Rubner: *Ztschr. f. Biol.*, xix, p. 545, 1883.

animals, and simultaneously Richet,<sup>3</sup> brought forth evidence to signify that the heat production of living animals was directly proportional to the surface of the body, thus apparently substantiating in every way the belief that the animal organism acted as a cooling body, and heat was produced to maintain the body temperature. Indeed, Rubner, citing his measurements of the heat production of several groups of animals, maintained that approximately 1000 calories of heat per square meter of body surface per twenty-four hours were given off by the living animal. Later Voit<sup>4</sup> concluded that the law of the constancy of heat production per square meter extended over a much wider range in the animal kingdom than had at first been believed.

This view of the intimate relationship between the surface area of the body and its heat requirement has had a strong hold upon European and American scientists for many years. This fact is emphasized by the regularity with which scientific research is presented in which the heat production is considered on the basis of per square meter of body surface. While the experimental evidence upon which this law was based was obtained exclusively in observations with animals, sufficient experimental evidence has been accumulated with human beings of varying ages, sex, and activities, to permit a critical discussion of the metabolism and the various factors affecting it to find if the laws of heat production per unit of body weight and of body surface are scientifically sound and if they obtain with the human organism.

> While the factor of body size is immediately recognized by all physiologists as a variant in studying basal metabolism, other possible factors are sex, age, muscular development, and the character of the preceding diet. In the seven years since the establishment of the Nutrition Laboratory, a definite investigation has been in progress to secure values for the basal metabolism of a large number of individuals of all ages for an ultimate analysis of causes and laws governing the heat lost from the body. Therefore in this discussion all other researches are disregarded simply because they were not made primarily with

<sup>3</sup> C. Richet: *Arch. de physiol.*, xvii, p. 284, 1885.

<sup>4</sup> E. Voit: *Ztschr. f. Biol.*, xli, p. 120, 1901.

a view to securing data of this kind, and it thus seems wisest and most logical to consider only those values which were secured primarily for this purpose with as nearly as possible like conditions, with similar technique, and with due regard to all the extraneous external factors known at the time to affect metabolism.

The data at present consist of observations upon eighty-nine men and sixty-eight women,<sup>5</sup> all of whom may be grouped as normal individuals, meaning thereby that they are people in apparently good health. In addition we have data for a large number of new-born infants<sup>6</sup> and a few infants under one year of age who would be distinctly classed as normal. A considerable number of observations are also available which were made upon infants who, though atrophic, were not otherwise abnormal.<sup>7</sup>

In the studies with adults, the two important factors affecting metabolism, namely, the influence of the ingestion of food and the influence of external muscular activity, were completely eliminated, the first by studying the subject only in the post-absorptive condition—that is, at least twelve hours after the last food—and the second by a graphic demonstration of complete muscular repose. This graphic record was obtained by one of the various forms of technique developed in this laboratory, in which the subject is either provided with one or two pneumographs about the chest and thighs, or lies upon a bed which records the slightest alterations in the center of gravity of the body. Our main problem, then, is to find what factors affect the quiet, resting metabolism, when the subject is in the post-absorptive condition.

*Total metabolism compared with total body weight.*

We may examine, first, the relationship between total metabolism and the body weight. Since we should expect that a large individual would produce more heat than a small individual,

<sup>5</sup> F. G. Benedict, I. E. Emmes, P. Roth, and H. M. Smith: this *Journal*, xviii, p. 139, 1914. The recently appearing results of W. W. Palmer, J. H. Means, and J. L. Gamble (*ibid.*, xix, p. 239, 1914) are accorded special notice.

<sup>6</sup> F. G. Benedict and F. B. Talbot: *Am. Jour. Dis. Child.*, viii, p. 1, 1914.

<sup>7</sup> Benedict and Talbot: *Carnegie Institution of Washington Publications*, No. 201, 1914.

it is of interest to see if a definite relationship exists between the body size and the total metabolism. Consequently, with the commonly used index of body weight as an index of size, the heat production of our subjects, both male and female, may be studied on the basis of the total heat production compared to the total weight. For this comparison it has been deemed most advantageous to plot the values for the eighty-nine men and sixty-eight women in the form of charts with the ordinates as total calories and the abscissae as body weight. These are given for the men in Chart I and for the women in Chart II. By thus separating the men from the women we take immediate cognizance of the fact that the two organisms may not advantageously be compared, as shown in the foregoing paper by Benedict and Emmes.<sup>8</sup> Likewise, in order to emphasize the fact that athletes<sup>9</sup> as a class may not be properly compared with non-athletes, the athletes on Chart I are marked by small crosses instead of dots. Finally, for purposes of comparison, we have added to these charts the values recently published by Palmer, Means, and Gamble<sup>10</sup> for a group of normal young men and normal young women, designating them by dots enclosed in circles. Special discussion of the athletes and the normal cases of Palmer, Means, and Gamble will enter into the consideration of all these data.

An examination of Chart I shows that, as would be expected, the men of small body weight have generally a much less total heat production than those of large body weight, and yet the relationship is by no means a clear one. Thus, of the numerous subjects with a total energy output of approximately 1600 calories per day, one at least has a body weight of less than 50 kgm., and another has a body weight close to 83 kgm., showing two organisms of widely varying weight producing essentially the same amount of heat per twenty-four hours.

It is of interest also to note the possible variations in the heat output of various individuals of the same body weight. Thus, with those weighing about 58 to 60 kgm. we have a variation from 1331 calories to 1748 calories, and with those weighing

<sup>8</sup> F. G. Benedict and L. E. Emmes: *this Journal*, xx, p. 253, 1915.

<sup>9</sup> F. G. Benedict and H. M. Smith: *ibid.*, xx, p. 243, 1915.

<sup>10</sup> Palmer, Means, and Gamble: *loc. cit.*

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about 80 kgm., the variation is from 1615 calories to 2126 calories, these representing the extremes. It is clear from Chart I that the athletes as a class have a very much higher metabolism than have the normal individuals of the same weight.

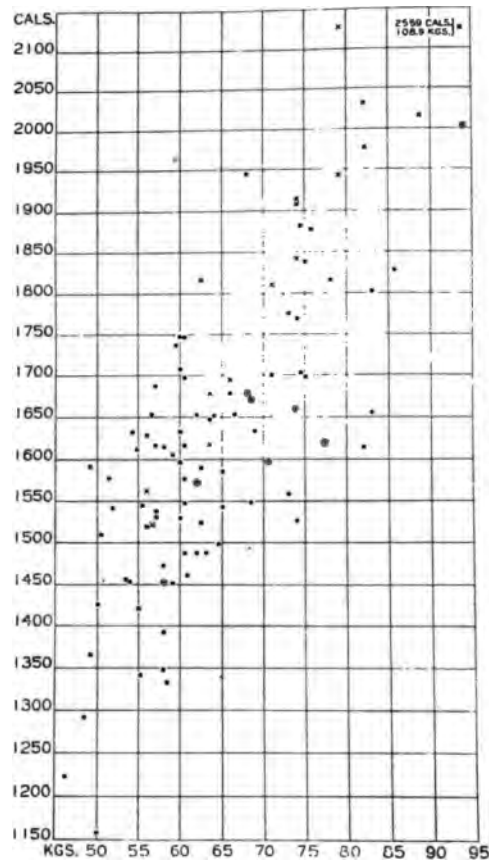


CHART I. Comparison of body weight and total twenty-four hour heat production of ninety-seven normal men.

In the observations of Palmer, Means, and Gamble, presumably great care was exercised to secure the basal metabolism of the individuals studied, and there is every indication that most careful attention was given to experimental technique. Their

values may therefore be taken as those which would be reasonably expected from a group of normal individuals. It will be seen that even in this small, selected group there was a considerable divergence in the heat production, and that it does not appear to be a function of the body weight.

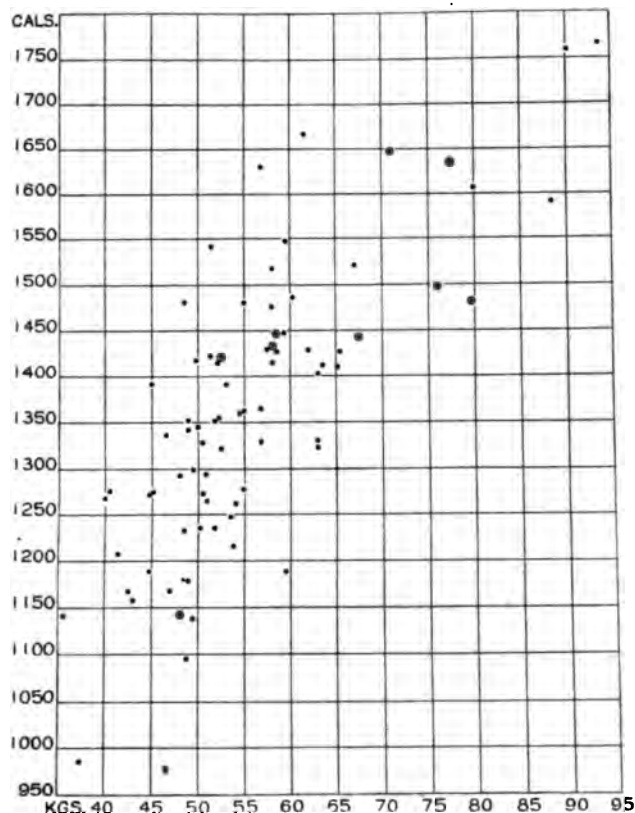


CHART II. Comparison of body weight and total twenty-four hour heat production of seventy-seven normal women.

If we consider the normal values for women which are plotted in Chart II, we find here also a general tendency for the heavier individuals to have the larger metabolism, although there is by no means a regularity in the relationship between body weight and the total metabolism. Thus, two women, having a total



heat output of 1475 calories, have as a matter of fact a body weight ranging from 48 to 80 kgm. Perhaps the most striking difference is that between two individuals having a body weight of approximately 60 kgm., the total heat production being respectively 1187 and 1666 calories. When we consider by themselves the values obtained by Palmer, Means, and Gamble, designated here also by dots enclosed in circles, we note likewise that individuals having approximately the same heat output, namely, 1450 calories, may have a difference of 27.5 kgm. in weight, with no approximation to a constant relationship between the total metabolism and the total body weight.

It is clear from an examination of Charts I and II that the relationship with both men and women between total body weight and total heat production is therefore only a most general one and the diversities in the values found are so great as to make it impossible to establish anything approximating a uniformity; thus we find here not the slightest evidence of a law governing the relationship between the total body weight and the total heat production.

*Heat production per kilogram of body weight.*

In the foregoing discussion the common index to size, namely, weight, has been utilized, the assumption being made that in this form of comparison there is tacit assent to the general conception that mere weight is the determining factor. It was early recognized, however, that it is somewhat illogical to compare the total heat output of two individuals varying widely in size, as, for instance, a small infant with that of an adult, and one of the earliest attempts to secure uniformity and a rational basis of comparison was the expression of the total metabolism in terms of heat per kgm. per twenty-four hours.<sup>11</sup> The extensive use of the heat production per kgm. of body weight by Continental and American writers makes it necessary for us to examine very closely the propriety of this procedure. The values for the metabolism measurements obtained with the eighty-nine men

<sup>11</sup> J. Forster: *Amtl. Ber. d. 50 Versammlung deutsch. Naturforscher u. Aertze in München*, Munich, 1877, p. 355. Forster used 10 kgm. of body substance as his unit.

and sixty-eight women previously referred to have therefore been plotted on this basis in Charts III and IV; the heat production per kgm. of body weight is given in the ordinates, and the body weight in kgm. in the abscissae.

The entire absence of uniformity with the men subjects is even more strikingly shown in Chart III than in Chart I. While there may be a slight tendency for the individual of the smallest

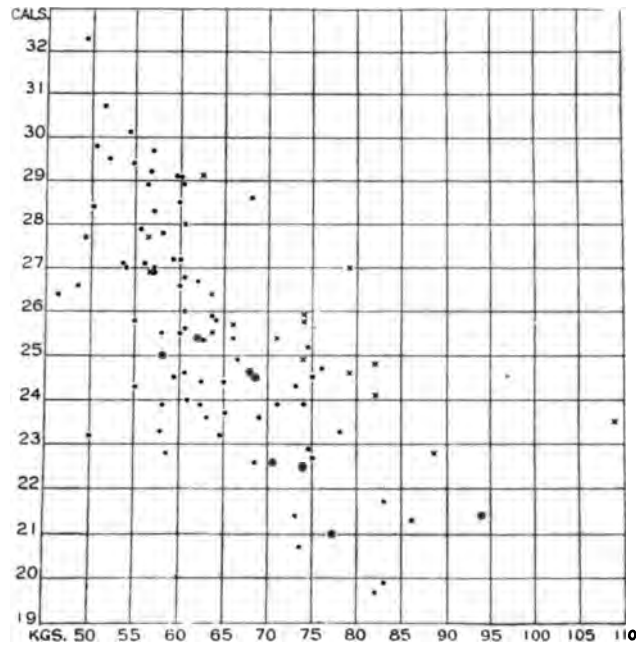


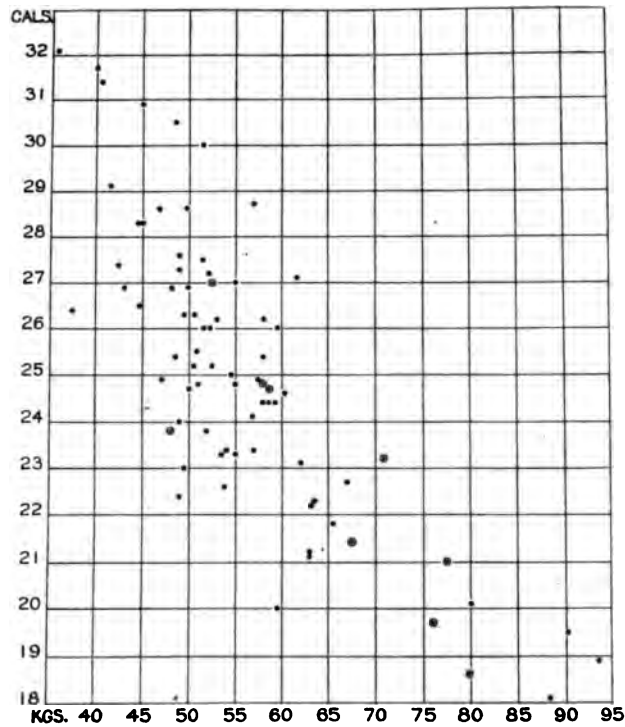
CHART III. Comparison of body weight and heat production per kgm. of body weight per twenty-four hours of ninety-seven normal men.

body weight to have the highest heat production per kgm. of body weight, on the other hand we have at least two individuals with a heat production of about 23.5 calories per kgm. of body weight, one of whom weighs 108 kgm. and the other 50 kgm., or less than one-half the weight of the first subject. Furthermore, of two individuals having very nearly the same body weight (50 kgm.), one shows a heat production of 23.2 calories per kgm. of body weight and the other 32.3 calories per kgm. of body weight.

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In other words, there is no possibility of finding a general relationship between body weight and the heat production per kgm. of body weight.

The values for the athletes show a tendency to group towards the upper side of the chart. Of special interest are the added values for the subjects of Palmer, Means, and Gamble, for even



• CHART IV. Comparison of body weight and heat production per kgm. of body weight per twenty-four hours of seventy-seven normal women.

these selected normal individuals of 20 to 30 years of age have a heat per kgm. of body weight ranging from 21.0 to 25.4 calories, with no tendency towards a constant relationship.

With the women subjects shown in Chart IV, the grouping is much the same as for the men subjects in Chart III. Although the values for the heat production per kgm. of body weight range from 18.1 calories to 32.1 calories, and, in general, the women

with the smallest body weight show a tendency to have the highest heat production per kgm. of body weight, there is nevertheless an absence of uniformity, particularly with the women whose body weights range from 50 to 60 kgm. The tendency towards a constant relationship is, however, much more evident than with the male subjects..

Of the comparisons thus far noted, it is probable that the group of women subjects studied by Palmer, Means, and Gamble come the nearest to approximating regularity; for while we find actual variations on the basis of per kgm. of body weight of 18.6 calories to 27 calories, nevertheless a probable line drawn through these points would imply that if the heat production per kgm. of body weight as well as the actual body weight were taken into consideration, there would be a semblance to regularity. Projecting the most probable straight line through these points, we find variations of plus or minus 8 to 10 per cent, variations still too great to permit this relationship to be classed as a physiological law.

With men we have found that the heat production per kgm. of body weight ranged from 19.7 calories to 32.3 calories, *i.e.*, a range of 60 per cent or more; with women from 18.1 calories to 32.1 calories, or a range of 80 per cent. With infants Benedict and Talbot found that the values varied from 42 to 88 calories, including both normal and atrophic infants; with the normal infants alone, they found a range in the heat production from 42 to 62 calories, or a difference of over 40 per cent. It is obvious, therefore, that any system of comparison which results in an error of 60 per cent with normal men, 80 per cent with normal women, and 40 per cent with normal infants cannot be considered as having the fundamental characteristics of a law, and that accordingly this system of notation should be discarded. If we apply this unit of measurement to the specially selected normal individuals of Palmer, Means, and Gamble, we find a variation of 21 per cent with the men and 45 per cent with the women. It is thus clear that the metabolism per kgm. of body weight has no claim to be considered as a physiological function, and its usage is accordingly no longer justified.

*Heat per unit of body surface.*

We have now to consider the second common unit for measurement of the heat production; namely, the heat production per unit of body surface. This, while fundamentally based upon Newton's law of cooling, and of the similarity in body surface of similar geometrical solids, actually calls for an elaborate study of the measurement of the body surface of man to establish a constant to be used in computation. It is known that the surface of irregular but similarly shaped bodies varies as the cube root of the square of the volume. By measuring a number of individuals, Meeh found that with adult man a constant of 12.312 should be employed in using this formula. Subsequent to the appearance of Meeh's paper,<sup>12</sup> his constant has been by common consent employed by numerous physiologists for computing the body surface of adults. The constant for infants was found by Meeh to be 11.9, although subsequent measurements by Lissauer<sup>13</sup> have shown that the value 10.3 is more nearly accurate. For the measurement of the metabolism of adults, the Meeh formula has been commonly used. Doubt has been thrown, however, upon the accuracy of this formula, and in a number of instances when a disagreement between Rubner's law of the constancy of the heat production per square meter of body surface has been observed, the popular method of explaining such irregularities has been to assume a disproportion between the body surface and the weight and an erroneous application of the Meeh formula. Rubner<sup>14</sup> himself has considered this with two boys of widely varying body composition, and showed a possible variation in the formula of about 7 per cent.

Employing the Meeh formula we have computed the body surface of all of our men and women subjects and plotted the values found for the heat production per square meter of body surface in Charts V and VI. With our men (Chart V) the values ranged from 693 calories per square meter of body surface per twenty-four hours to as high as 958 calories per square meter of body surface. In examining this chart it should be taken into consideration that according to the law which has been used by

<sup>12</sup> K. Meeh: *Ztschr. f. Biol.*, xv, p. 425, 1879.

<sup>13</sup> W. Lissauer: *Jahrb. f. Kinderheilk.*, lviii, p. 392, 1903.

<sup>14</sup> Rubner: *Beiträge zur Ernährung im Knabenalter mit besonderer Berücksichtigung der Fettsucht*, Berlin, 1902, p. 40.

physiologists for many years as a basis for comparison, all the points would be expected to fall in a horizontal line. Even with the selected subjects of Palmer, Means, and Gamble, the values range from 726 calories to 818 calories, a variation—ap-

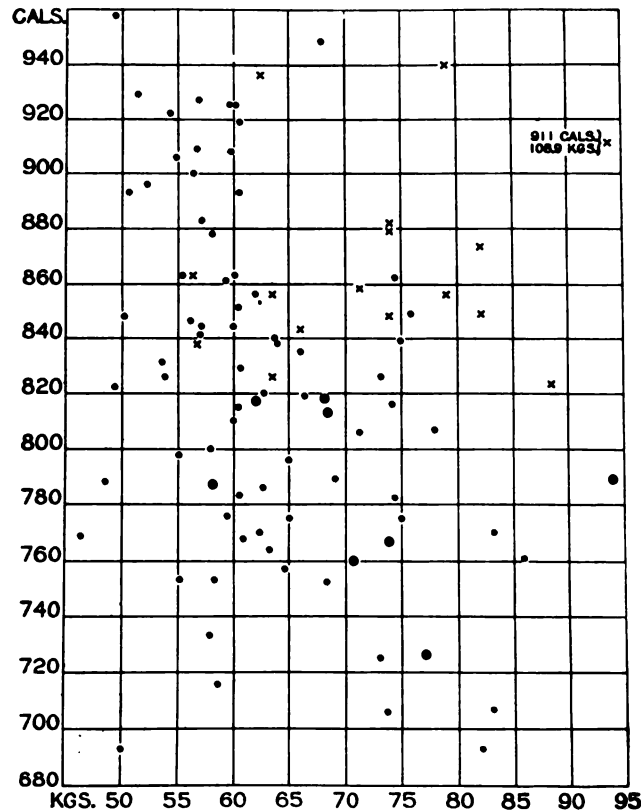


CHART V. Comparison of body weight and heat production per square meter of body surface per twenty-four hours of ninety-seven normal men.

proximately 12 per cent—which would hardly designate this supposed relationship as a physiological constant.

In a recent paper Du Bois<sup>13</sup> has maintained that an average

<sup>13</sup> E. F. Du Bois: *Jour. Am. Med. Assn.*, lxi, p. 827, 1914. In this connection attention should be called to the attempts of D. and E. F. Du Bois to improve upon the method of computing the body surface by a series of carefully selected measurements (D. and E. F. Du Bois: *Arch. Int. Med.*, xv, (in press) 1915).

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of a selected number of normals, from which he has excluded athletes (a debatable procedure) and all individuals other than those of perfectly normal proportions, has given an average value of 34.2 calories as the heat production per square meter per hour, or 821 calories per square meter per twenty-four hours. A large number of our so called "normal" subjects would be

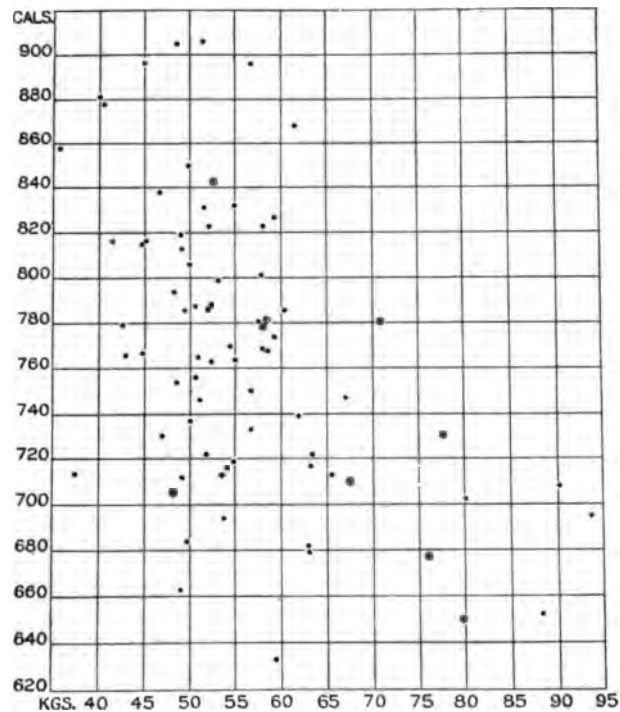


CHART VI. Comparison of body weight and heat production per square meter of body surface per twenty-four hours of seventy-seven normal women.

excluded by Du Bois from his classification on account of their being athletes or people of unusual shape, but the values found by Palmer, Means, and Gamble were for the most part secured with individuals of such size and weight as would fully conform to Du Bois's specifications. Yet we find that all the values given by Palmer, Means, and Gamble fall below the Du Bois figure;

indeed, one value falls 10 per cent below. We also find that all the athletes among our subjects are without exception above the value of 821 calories, although but two are more than 10 per cent above this value.

Examining the plotted values for women (Chart VI) we find the range to be from 633 calories per square meter to as high as 905 calories per square meter, with no tendency for the values to fall in a horizontal direction or to average at any given value. With the normal women of Palmer, Means, and Gamble, we likewise find an extensive range, namely, from 650 calories to 843 calories per square meter of body surface, with no approach to constancy in the arrangement of values.

The gross irregularities in these plots agree wholly with the variations found for infants by Benedict and Talbot. Including all the infants studied, the values for the heat production per square meter of body surface ranged from 554 calories to 1334 calories; the strictly normal infants varied from 554 calories to 998 calories.

It is obvious that any basis of comparison which involves possible variations of 40 per cent with men, of 43 per cent with women, and 80 per cent with normal infants, cannot be considered as a physiological law. If a special selection is made, as was done by Du Bois, it may be possible so to select a certain group as to approximate constancy. It seems particularly unfortunate, however, that the normal individuals who are of the greatest value in comparisons with pathological cases are not those of normal height and weight. The majority of pathological individuals are either above or below weight, and hence it is necessary to use for comparison only normal individuals who lie outside of the supposed regular or normal shape of individuals. For instance, it is obviously unfair to compare an emaciated diabetic<sup>18</sup> with a normal, well nourished man. A man who was thin and of the same height and weight as the emaciated diabetic would, by the process of selection above referred to, be excluded from a normal grouping as being impossible to use. Even the selected groups studied by Palmer, Means, and Gamble show differences between the lowest and highest values of about 12 per cent for the men and 30 per cent for the women.

<sup>18</sup> F. G. Benedict and E. P. Joslin: *Carnegie Institution of Washington Publications*, No. 176, p. 120, Table 131, 1912.



If, however, we are to consider that surface area of the body determines the resting metabolism, we have no right to apply this law solely to a selected group of normal individuals. It must, if it is a physiological law, apply as well to a new-born infant, to an atrophic infant, or to an athlete. Large variations in the heat production per square meter of body surface are noted with these groups, variations which can in no wise be explained by the widest assumption of a discrepancy in the relationship between the body weight and the body surface or of an error in the commonly used formulae for this computation. It must furthermore be recognized that while, with selected normal individuals, a constancy approximating plus or minus 10 per cent may be said to obtain, there is grave danger in laying great stress upon this apparent agreement; for it may be considered as a fundamental tenet of physiological experimentation that, even among normal individuals, Subject A may not be compared to Subject B even if the two are of the same height and weight. On the other hand, in studying disturbances of metabolism as a result of disease or of a superimposed factor, it is perfectly permissible to compare a group of fifteen or more persons with an equal number of normal individuals of the same height and weight; for, in such a group comparison, the same errors which may enter into the application of a formula for computing body surface from body weight would obtain with the two sets of individuals compared. An excellent illustration of this may be found in the observations in this laboratory on diabetics in which, by applying the group system, an increment in the basal metabolism amounting to approximately 20 per cent was noted in severe diabetes.

That there is with normal individuals a rough approximation between basal metabolism and body surface is not surprising in view of the recent investigations on growth.<sup>17</sup> If the blood volume, area of the trachea, and area of the aorta have been found to bear a simple mathematical relationship to the total body weight in normal individuals—a relationship expressed by the cube root of the square of the body weight—it is not unlikely

<sup>17</sup> G. Dreyer and W. Ray: *Phil. Trans., Series B*, cci, p. 133, 1909-10. G. Dreyer, W. Ray, and E. W. A. Walker: *Proc. Roy. Soc., Series B*, lxxxvi, pp. 39 and 56, 1912 and 1913.

that the active protoplasmic tissue of the body, *i.e.*, the mass of heat-producing matter, as well as the surface will have approximately the same mathematical relationship. Furthermore, if the heat production is proportional to the cube root of the square of the weight, it is due not to the fact that surface area determines thermolysis, but that the mass of active protoplasmic tissue, with probably the same mathematical relationship to the body weight, determines thermogenesis.

It is clear, therefore, that even with normal individuals a relationship between body surface and heat production which may be expressed with any approximation to mathematical accuracy does not exist. Hence, in considering the metabolism of normal men and women, we are compelled to maintain that the so called "law" of heat production per square meter of body surface does not obtain.

*Relationship between heat production and body composition.*

The two laws supposed to determine the metabolism in the human individual, *i.e.*, the law of body weight and the law of body surface, both assume the simplest relationship between the size of the body and its heat-producing mechanism. Thus, in the law of body weight the natural assumption is made that each kgm. of body material has the same heat-producing power as every other kgm. of body material; in other words, that there is uniformity in heat production throughout the whole body. The second law, *i.e.*, that the heat requirement of the body is directly proportional to its area, assumes that the basal heat loss of all individuals is constant per square meter of surface. These beliefs have so strong a hold upon modern writers that but few have given serious thought to the possibility of there being changes in the *mass* of active protoplasmic tissue in different bodies of the same size. The modern conceptions of the seat of combustion in the living body make almost unnecessary the assumption that heat production is greater in muscular tissue than in fatty tissue, and yet it is seemingly tardily admitted that inert body fat must be considered in a different category as to the function of heat production than either muscular or glandular tissue. Once this is admitted, however, the inadequacy of the

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computation of the heat production per kgm. is obvious. Formerly the bones were also classified as inert material, but more recently McCrudden<sup>18</sup> has maintained that the bones are fully as active in metabolism as are the muscles and the glands.

Again it has been commonly considered in all the literature that the active protoplasmic tissue or the heat-producing organism of the body works with a constant intensity. In other words, little if any consideration is given to the possibility of there being various metabolic levels, or that the heat-producing organism *per se* may functionate with a greater or less intensity.

It is proper for us to consider here, therefore, first, what is the influence upon the basal metabolism of changes in the body composition or changes in the relative proportion of active protoplasmic tissue and inert body fat, and second, what are the stimuli to metabolism, noting if possible what variations in metabolism may be found in an organism which has essentially a constant mass of active protoplasmic tissue and a constant body surface, but with varying intensity of stimulus.

*Effect of variations in the mass of active protoplasmic tissue.*

Variations in the proportions of the protoplasmic tissue and fatty tissue in the body may be expected with athletes, with men as compared with women, with men and women of similar weight but different height, and with certain infants.

It is a well known fact that the process of athletic training removes inert body fat and increases and hardens the muscular tissue, resulting in a greater proportion of protoplasmic tissue in the body. The study in the foregoing paper by Benedict and Smith<sup>19</sup> shows clearly that with trained athletes, particularly with the heavier men, the basal metabolism according to the three standards of comparison, namely, the total metabolism, the heat production per kgm. of body weight, and the heat production per square meter of body surface, is measurably greater with athletes than with non-athletic individuals of the same weight and height. Thus we have the first clear index as to the

<sup>18</sup> F. H. McCrudden: *Transactions of the XVth International Congress on Hygiene and Demography*, Washington, ii, p. 424, 1913.

<sup>19</sup> Benedict and Smith: *loc. cit.*

definite influence of a greater proportion of muscular tissue upon the total metabolism.

It is, furthermore, a well known fact that men as compared with women have a greater muscular development and are capable of longer and more enduring muscular activity; consequently they do not, as a rule, have an excess of subcutaneous fat. In comparing a man and a woman of the same height and weight, therefore, one would normally expect to find a larger proportion of protoplasmic tissue with the man than with the woman. The observations of Benedict and Emmes have shown clearly that when such comparisons are made, there is a distinct, although not necessarily striking, difference between the two sexes, the men having the greater metabolism.

In the report of the study of the metabolism of normal and atrophic infants, Benedict and Talbot have pointed out that with two infants of the same height and weight, the older infant, who would naturally be atrophic, would frequently have the greater proportion of active protoplasmic tissue, while the younger would have the larger proportion of fat. It was found that the basal metabolism of the atrophic infant was always higher than that of the normal child.

In comparing adults it is safe to state that with two individuals having the same weight but a difference in height, the taller individual will have the greater proportion of active protoplasmic tissue, and the shorter the larger proportion of fat. We have therefore compared in Table I a number of individuals of different heights, but of the same weight. Anticipating the discussion in a succeeding section of the influence of age upon the metabolism, we have also selected for this comparison only those individuals who are approximately of the same age. Unfortunately the data for our eighty-nine male subjects, extensive though they are, do not permit a large number of comparisons of individuals having the same weight and age with widely varying heights; but such evidence as is given in Table I shows that the taller person has the larger heat production.

In the comparison of normal infants with atrophic infants, of athletes with non-athletes, and of men with women, we have used individuals of like height and weight, and find a difference in the heat production of the two classes compared. In our

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comparison of men of the same weight and age but widely varying heights, we have also found that the taller individual has a greater heat production than the shorter individual of the same weight. It is only reasonable to suppose that in all these comparisons we deal with actual differences in the total proportion of active protoplasmic tissue. The variations in the heat production may, however, be due, at least in part, to the fact that the active protoplasmic tissue functionates with a varying degree of intensity.

TABLE I.

*Comparison of the heat production of normal men of like age and weight but of different height, in experiments without food.*

SUBJECT	AGE	NO. OF PERIODS	HEIGHT	BODY WEIGHT		TOTAL HEAT (COMPUTED) PER 24 HRS.
	YRS.		CM.	KG.	LB.	
F. G. B.	41	37	183	83.1	180.2	1802
Prof. C.	36	12	169	83.0	180.5	1655
K. H. A.	26	110	182	66.4	146.5	1654
F. P. R.	22	58	173	65.1	143.5	1543
W. G. J.	21	26	175	60.5	133.3	1746
S. A. R.	23	44	165	60.8	134.0	1460
R. I. C.	26	9	184	56.8	125.3	1687
A. G. E.	26	68	169	57.0	125.7	1531
D. J. M.	20	31	175	58.0	128.0	1615
E. T. W.	22	12	169	57.8	127.5	1472

### *Effect of variations in stimulus.*

All comparisons of basal metabolism thus far made assume that the cellular activity of the protoplasm is constant in all cases, and the possibility of there being material differences in the metabolic level or the cellular activity under different conditions has not hitherto been given proper consideration.

One of the first suggestions of a variation in the intensity of cellular activity is noted in Chart V, which shows the metabolism of our men subjects per square meter of body surface. In this chart we find that with eight men, weighing approximately 80 kgm., the heat production per square meter of body surface ranged from 693 to 940 calories, and that five men, weighing 50 kgm. or under, ranged from 693 to 958 calories. In other

words, the two extremes of the heavy fat men and the thin small men show a wide range in the metabolism per square meter of body surface. This points strongly towards distinct differences in the intensity of cellular activity with different individuals rather than to corresponding mathematical differences in the mass of active protoplasmic tissue in the several groups, least of all to possible disturbances in the relationship between body weight and computed body surface.

If we seriously consider the question of the possible stimuli to metabolism we find that it brings us immediately to a large number of possible variations in the metabolic intensity of normal individuals. While the evidence presented in the preceding papers indicates differences in basal metabolism due to sex and to the muscular development of athletes, the popular conception that the vital activities of youth are greater than those of old age is certainly not without some foundation, and hence we should consider the influence of age upon the metabolism.

#### *Effect of age.*

Benedict and Talbot in their comparison of normal and atrophic infants have pointed out the influence of age upon the metabolism, but in comparing adults we have to consider not the effect primarily upon the proportion of muscle and fat, but the influence of age upon the body vigor and the cellular activity. In examining critically all the foregoing charts, we find that those individuals showing widest variations from the general course are somewhat frequently found among those over 32 years of age and those under 18 years of age. Furthermore the youths are grouped in a different part of the charts from the older men.<sup>20</sup> While this was not invariably the case, yet we were sufficiently impressed with the fact to study more into the effect of age upon basal metabolism. Unfortunately the results of the observations made in this laboratory, while very numerous, do not con-

<sup>20</sup> The number of plots on each chart is so great as to preclude indicating the initials of all individuals, but usually from the data in the charts and the large general table of Benedict, Emmes, Roth, and Smith (*loc. cit.*), no difficulty will be experienced in recognizing the individual points. This is also true for the subjects of Palmer, Means, and Gamble.

TABLE II.

*Comparison of the heat production of normal men and women of like body weight and height but of different age, in experiments without food.*

MEN										WOMEN									
GROUP AND SUBJECT		AGE	NUDE WEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)			AGE	NUDE WEIGHT	HEAT PRODUCTION PER 24 HRS. (COMPUTED)										
				Total	Per kgm.	Per square meter			Total	Per kgm.	Per square meter								
Group I										Group I									
F. M. M.	16	59.7	173	1739	29.1	925	16	56.8	157	1630	28.7	896							
W. W. C.	17	56.3	172	1629	28.9	900													
Average.						29.0	913												
T. H. Y.	22	59.2	169	1605	27.2	861	31	53.6	155	1247	23.3	713							
E. T. W.	22	57.8	169	1472	25.5	800	36	54.9	153	1276	23.3	719							
J. C. C.	22	56.1	173	1522	27.1	846	38	59.5	159	1187	20.0	633							
J. K. M.	24	60.4	173	1549	25.6	815	Average.						22.2	688					
H. L. H.	26	60.5	172	1696	28.0	893	Group II												
A. G. E.	26	57.0	169	1531	26.9	841	Miss R. M.	16	52.1	162	1353	26.0	787						
L. E. E.	31	59.8	175	1707	28.5	908	Miss M. C.	16	50.6	162	1273	25.2	736						
A. L.	40	60.6	171	1576	26.0	829	Average.						25.6	772					
Average.						26.9	849	Miss F. E.	22	53.1	162	1391	26.2	799					
Group II										Miss G. J.	22	49.7	160	1139	23.0	684			
V. G.	17	54.3	162	1632	30.1	922	Miss K. M.	22	48.2	161	1294	26.9	794						
F. P.	17	49.3	161	1591	32.3	958	Miss C. B.	24	49.8	162	1419	28.6	850						
Average.						31.2	940	Miss M. S.	24	48.5	159	1480	30.5	905					
										Miss E. C.	25	50.5	164	1327	26.3	788			

M. B. ....	27	53.6	160	1455	27.1	831	Miss J. B. ....	27	51.1	163	1265	24.8	746
T. M. C. ....	35	48.5	165	1292	26.6	788	Mrs. C. E. ....	74	48.9	164	1095	22.4	663
Dr. P. R. ....	41	55.2	164	1341	24.3	753	Average.....					26.1	779
Average.....					26.0	791							
<i>Group III</i>													
O. F. M. ....	24	85.8	171	1827	21.3	761	Miss I. B. ....	18	50.1	166	1235	24.7	737
							Miss L. ....	25	52.4	168	1321	25.2	763
Prof. C. ....	36	83.0	169	1655	19.9	707	Miss E. C. ....	25	50.5	164	1327	26.3	788
H. F. ....	63	82.1	166	1615	19.7	693	Miss J. B. ....	27	51.1	163	1265	24.8	746
Average.....					19.8	700	Miss A. D. ....	37	52.3	166	1355	26.0	788
							Mrs. C. E. ....	74	48.9	164	1095	22.4	663
							Average.....					24.9	760
Average, younger subjects.....					27.2	871							
Average, older subjects.....					24.2	780	<i>Group IV</i>						
							Miss E. W. ....	24	40.5	157	1273	31.4	878
							Mrs. S. C. ....	52	37.4	155	985	26.4	714
							Average, younger subjects.....					27.6	821
							Average, older subjects.....					24.9	733



tain so large a number of people in middle age or old age or, indeed, in early youth as we would like. Nevertheless, there are a certain number of comparisons available and a few are given in Table II which permit the study of the influence of a difference in age upon the metabolism of individuals with the same body weight and height.

It will be noted with the men subjects that in all cases the youths had a very much higher metabolism than the adults with whom they were compared. Thus, in the first group the two boys showed a considerably higher metabolism than that of the adults in the same group. In Group II both boys showed a very much higher metabolism than the other two subjects, while in Group III the heavy young man, O. F. M., 24 years old, showed a perceptibly higher metabolism than the two older men 36 and 63 years, respectively, with whom he was compared. The average of all the younger subjects both per kgm. of body weight and per square meter of body surface was considerably higher than that of the older subjects.

With the women subjects the difference was not so sharply defined. In Group I the 15 year old girl had a much higher metabolism than the other subjects; in Group II the metabolism of the girls was essentially the same as that of the women. In Group III the 18 year old girl had a metabolism slightly lower than the average of the women in the group with whom she was compared, but in Group IV the 24 year old woman had a much more active metabolism than the 52 year old woman with whom she was compared. Here again, the general average, defective though it obviously is, shows a distinctly greater metabolism for the younger subjects.

This difference in metabolism of individuals of different ages was early noted by Sondén and Tigerstedt,<sup>21</sup> who conclude that age and especially the age of growth has a great influence upon the total metabolism of the body, and that the younger organism has the greater metabolism per unit of body surface than has the older.

Similarly Magnus-Levy and Falk<sup>22</sup> noted that the metabolism

<sup>21</sup> K. Sondén and R. Tigerstedt: *Skand. Arch. f. Physiol.*, vi, p. 218, 1895.

<sup>22</sup> A. Magnus-Levy and E. Falk: *Arch. f. Anat. u. Physiol., Physiol. Abt.*, Supplement, p. 314, 1899.

of children is greater than that of adults and that the metabolism decreases in old age. This finding likewise applied to the measurements on the basis of heat per square meter of body surface.

It is somewhat difficult to differentiate sharply between the influence of variations in the total proportion of active protoplasmic tissue and the influence of variations in the stimulus to the cellular activity, and to say, for example, whether in old age the lower metabolism is due to a lower cellular activity or to an actual decrease in active protoplasmic tissue from disintegration and loss and atrophy of the tissues. A fact of fundamental importance, however, is that there may be, certainly in the cycle of a lifetime, very considerable changes in the metabolism of an individual. The youth has a higher metabolism than the person in middle life, while one of advanced years has a still lower metabolism than the person in middle life. Thus far the history of investigations in metabolism has been written in too short a time to enable an accumulation of accurate scientific data concerning the metabolism of the same individual during various periods of life. Practically no investigations include a study of the metabolism of a subject in early youth as compared with his metabolism in middle life or later.

It is not necessary, however, to await the entire period of a lifetime to note differences in the intensity of cellular activity; for there are numerous factors which may produce in a short time changes in metabolism comparable to those noted with people of different ages. Among these may be mentioned sleep, character of diet, and after-effects of severe muscular work.

#### *Influence of sleep.*

It is possible to maintain complete muscular repose and yet have the brain active and awake; on the other hand, we may have complete muscular repose with the subject sleeping. Observations on deep sleep made in earlier investigations have led to the almost unanimous belief that sleep has no influence upon the metabolism.

A study of prolonged fasting recently carried out in this laboratory afforded an excellent opportunity for comparing the metabolism during the night when the subject was sleeping quietly

in the bed calorimeter with that of the next morning when he was lying quietly upon the same bed, awake and breathing into the universal respiration apparatus. The subject slept for the greater part of the period of observation in the bed calorimeter, the graphic record of the body movements made by the self-recording bed showing that the man was remarkably quiet throughout the whole night. During the morning observation, when the subject was connected with the respiration apparatus, he was phenomenally quiet, the graphic record showing a practically straight line in every experiment. According to the opinion of Mr. T. M. Carpenter, who made the observations with the respiration apparatus, the subject had the most complete muscular relaxation and control of any of the individuals that he had ever studied. The details of the observations with this subject may be found in the report of this fasting experiment,<sup>23</sup> but it may be stated here that during the thirty-one days of fasting the metabolism gradually decreased. Without taking account of the changes in the body weight, we may compare for each day the metabolism of the subject while in the bed calorimeter during the night with his metabolism immediately afterward when he was connected with the respiration apparatus in the morning. These comparisons, which are given in Table III on the basis of the oxygen consumed, show in general an increase of 13.2 per cent for the morning metabolism when the subject was connected with the respiration apparatus.

The increased metabolism during the morning observations cannot be attributed to muscular activity; for a comparison of the graphic records shows that the degree of muscular repose was more nearly perfect in the morning experiments with the respiration apparatus than in the night experiments with the bed calorimeter, since it was naturally impossible for the subject to lie absolutely quiet throughout the whole night, even during sleep.

There is no question of the influence of food in the alimentary tract; for during the entire period of thirty-one days the subject ate absolutely no food and drank only about 900 cc. of distilled water per day.

<sup>23</sup> F. G. Benedict: *Carnegie Institution of Washington Publications*, No. 203, 1915.

TABLE III.

*Increase in metabolism of subject awake as compared with metabolism of subject asleep.*

DAY OF FAST	OXYGEN CONSUMED PER MIN			
	(a) Subject asleep	(b) Subject awake	Increase	
			(c) Amount (b-a)	(d) Per cent (c-a)
	cc.	cc.	cc.	
1st	196	237	41	20.9
2d	208	227	19	9.1
3d	198	226	28	14.1
4th	187	212	25	13.4
5th	176	205	29	16.5
6th	185	200	15	8.1
7th	185	204	19	10.3
8th	177	203	26	14.7
9th	173	190	17	9.8
10th	179	187	8	4.5
11th	166	187	21	12.7
12th	173	187	14	8.1
13th	167	192	25	15.0
14th	160	181	21	13.1
15th	162	179	17	10.5
16th	158	182	24	15.2
17th	154	182	28	18.2
18th	154	174	20	13.0
19th	153	177	24	15.7
20th	159	173	14	8.8
21st	137	174	37	27.0
22d	153	170	17	11.1
23d	144	165	21	14.6
24th	152	167	15	9.9
25th	147	166	19	12.9
26th	151	168	17	11.3
27th	145	172	27	18.6
28th	145	166	21	14.5
29th	152	171	19	12.5
30th	147	166	19	12.9
31st	148	166	18	12.2
Average				13.2

Since, therefore, the subject was asleep for the most part of the calorimeter observations in the night period and awake during the observations with the respiration apparatus in the morning period, and the metabolism was not influenced by muscular activity or the ingestion of food, it is logical to conclude that the increased metabolism during the morning observations was due to the fact that the subject was awake. The experimental data therefore justify the conclusion that deep sleep lowers perceptibly and very considerably the basal metabolism, and we may properly ask if our standard for the measurement of the basal metabolism is the correct one. We state as the only prerequisites of the measurement a post-absorptive condition and complete muscular repose, thereby eliminating the influence of the ingestion of food and the influence of external muscular work. Is it not conceivable that we should logically eliminate the question of increase in the internal muscular activity incidental to the waking condition, and consider the basal metabolism to be that obtaining during the post-absorptive condition, with complete muscular repose, and in deep sleep?

*Variations in metabolism as observed at different times.*

While it has thus far been impracticable to make studies of the same individual during early youth, middle age, and old age, it is important for us to note the significant changes in metabolism which may be observed with the same individual on consecutive days. As a result of observations by Zuntz and some of his students and the earlier published reports of Benedict and Carpenter, it has been commonly considered that the metabolism of an individual remains essentially constant from day to day. In the accumulation of the experimental data for this study, opportunities occurred to note the actual variations in the metabolism of individuals under the predetermined conditions of the post-absorptive condition and complete muscular repose. With many of these subjects observations were made on five or more days and frequently over periods of several months and even years. The results for a large number of our subjects who have been studied five days or more have been gathered together in Table IV.

TABLE IV.

*Variation in post-absorptive metabolism in experiments with normal men.*

SUBJECT	AGE	OBSERVATIONS		TIME COVERED BY EXPERIMENTS	VARIATION IN OXYGEN ABOVE MINIMUM
		Days	Periods		
	<i>yrs.</i>				<i>per cent</i>
F. G. B.	41	8	37	2 yrs. 2 mos.	10.8
E. G.	20	6	11	1 yr.	11.2
C. B. S.	26	26	75	1 yr. 2 mos.	18.2
J. H. H.	25	5	13	4 mos.	14.1
K. H. A.	26	25	110	11 mos.	19.3
J. R.	27	12	57	3 mos.	21.2
M. A. M.	29	53	157	4 mos.	21.3
F. P. R.	22	20	58	3 mos.	15.2
J. J. C.	27	53	252	2 yrs. 3 mos.	12.1
D. M.	22	5	15	5 dys.	15.3
M. J. S.	24	13	42	19 dys.	13.6
M. Y. B.	20	6	12	6 dys.	8.1
W. F. M.	21	7	12	15 dys.	16.7
H. H. A.	22	28	81	1 yr. 2 mos.	24.8
S. A. R.	23	9	44	5 mos.	16.6
W. G. J.	21	9	26	1 mo.	10.6
H. L. H.	26	35	120	2 yrs. 5 mos.	25.9
J. E. F.	21	7	24	5 mos.	22.7
J. K. M.	24	27	103	1 yr. 2 mos.	8.2
J. B. T.	20	11	43	8 mos.	8.9
W. F. B.	32	5	16	4 mos.	9.3
H. B. L.	20	5	18	2 mos.	8.6
L. E. E.	31	31	144	2 yrs. 1 mo.	19.8
Dr. S.	43	5	13	12 dys.	4.9
D. J. M.	20	5	31	2 mos.	7.7
H. F. T.	32	41	211	8 mos.	31.3
P. F. J.	20	18	82	9 mos.	10.0
A. G. E.	26	14	68	1 yr.	7.7
W. A. S.	21	5	10	2 mos.	5.4
Dr. P. R.	41	9	33	7 mos.	11.6
C. H. H.	19	9	25	4 mos.	12.1
V. G.	17	17	71	5 mos.	15.9
J. J. G.	21	6	20	2 mos.	9.6
T. M. C.	35	17	93	4 yrs. 5 mos.	14.9
J. H.	26	6	12	12 dys.	3.5
Average (35 subjects)					13.9

The metabolism in all cases was most carefully determined, so that variations in the results were not due to errors in technique, but to variations in the basal metabolism under the experimental conditions outlined. In the experiments with the respiration apparatus a tendency to fall asleep has been shown by many subjects, the sound of the motor and blower producing a soothing effect; consequently it is not possible to state that in every experimental period there was complete consciousness on the part of the subject. In a large majority of the experiments, however, the subject was fully awake and the variations noted between the maximum and minimum metabolism cannot fairly be stated as due to sleep.

As an index of the variations in metabolism during the time covered by the experiments, we have taken the increase in the oxygen consumption, using the minimum value as a basis. With these subjects it will be seen that in one case the oxygen consumption varied as widely as 31.3 per cent, while with still another subject it varied only 3.5 per cent. A general inspection of the data will show that, as a rule, the greatest variations were found with the subjects studied over the longest periods. While it is hardly correct to obtain an average value for the oxygen consumption for so many different individuals with such wide differences in the time covered by the experiments, yet such a value has been found and shows that on the basis of these observations there may be an average variation of 13.9 per cent in the basal metabolism, when measured in the post-absorptive condition and with complete muscular repose, during a period of two years or, in the majority of cases, considerably less. With no attempt to analyze the causes of these differences, it is sufficient here simply to call attention to their magnitude.

Examination of Table IV shows clearly the error of assuming, as is frequently done in metabolism experiments, a basal value for any one individual to compare with metabolism measurements made with a superimposed factor. Since this is illogical in the case of one individual, it is even more illogical to consider possibilities of a standard normal value for any group of individuals.

It is relatively rare for the metabolism of an individual to be determined for the same periods on consecutive days, and hence

it is difficult to state whether the stimulus to the cellular activity fluctuates very considerably from day to day or whether the variation in the metabolism is due to periodicity, weather, temperature conditions and changes, or similar factors. One of the most extensive series of observations in which the basal metabolism of an individual was determined practically every day is that reported by Benedict and Cathcart<sup>24</sup> with a professional athlete, M. A. M. With this subject the basal metabolism was determined almost daily for the period from December 7, 1911, to February 29, 1912, with a few observations after that date. Certain of the experiments were complicated by the fact that on the preceding day there were considerable variations in the character and nature of the diet, as the subject was given on some days a diet with but 100 grams of carbohydrate and on other days a diet with 400 grams. Excluding these days with special diet, we find the body weight varied from 64.5 kgm. to as high as 67.2 kgm. Excluding, also, the first day of experimentation, on which the conditions were admittedly unusual, we find during this period a minimum carbon dioxide production of 191 cc. per minute and a maximum production of 232 cc. per minute. The oxygen consumption during the same period ranged from a minimum of 225 cc. per minute to a maximum of 262 cc. per minute. The average values were 206 cc. per minute for the total carbon dioxide production, and 240 cc. per minute for the total oxygen consumption.

In this connection we may also refer to the recent article of Palmer, Means, and Gamble, which gives the results of a series of observations on W. W. P. for six days, from July 21 to July 26, 1914. The agreement of the values is as close as one could expect, and taking into consideration these values alone, one could readily assume a constant metabolic activity from day to day. On the other hand, if we examine the values obtained with W. W. P. in the winter of 1913-1914, which are given in the first general summary table of the article by Palmer, Means, and Gamble (their Table I), we find marked differences from the values secured in the summer of 1914. In the winter the body weight was essentially the same as in the summer, namely,

<sup>24</sup> F. G. Benedict and E. P. Cathcart: *ibid.*, No. 187, p. 78, 1913.



93.9 kgm. as compared with an average of 93.7 kgm.; but in the winter the total heat production was 2004 calories and in the summer 1797 calories; the heat production per kgm. of body weight was 21.4 calories in the winter and 19.2 calories in the summer; and the heat production per square meter of body surface was 789 calories in the winter and 707 calories in the summer. Thus in this most recent research we have indications of a marked difference in the basal metabolism as determined in the post-absorptive condition and with complete muscular rest. It should be stated, furthermore, that the series of experiments made in the winter of 1913-1914 was not the first made with W. W. P. with this apparatus, and the results therefore represented the metabolism of a more or less trained subject. Obviously he did not change in height in the period intervening between the two series, and, as has been shown, the weight did not change. His habits of life were such that probably there was no replacement of active protoplasmic tissue by inert fat. We are consequently forced to the conclusion that we have here not alterations in the amount or proportion of the protoplasmic tissue, but a distinct variation in the stimulus to cellular activity.

It should not be lost sight of that the fact that W. W. P. had a higher metabolism in the winter of 1913-1914 than in the summer of 1914 may be taken by some investigators as an indication of a larger metabolism due to an increased cooling of the body surface, and that we have here the possibility of a greater temperature difference with a greater metabolism. As a matter of fact, the temperature of the laboratory during both series of experiments was essentially constant, the difference being but a relatively few degrees. Furthermore, it is well known that the human body does not react to differences in temperature environment as do the lower animals. Thus the evidence of both Loewy<sup>25</sup> and Johansson<sup>26</sup> is strikingly against there being any material alteration in the metabolism of man with cold until the temperature difference is sufficiently great to induce internal muscular work due to shivering.

<sup>25</sup> A. Loewy: *Arch. f. d. ges. Physiol.*, xlv, p. 189, 1890.

<sup>26</sup> J. E. Johansson: *Skand. Arch. f. Physiol.*, vii, p. 123, 1897; xvi, p. 88, 1904.

Preliminary observations have been made upon dogs in this laboratory to note exactly the influence of temperature environment upon the metabolism. When the animals are suspended in a crib or cage, by means of which a graphic record can be obtained of the muscular activity, it has been found that with no muscular movement or shivering, very much greater differences in temperature environment may be borne without change in the metabolism than had hitherto been supposed. In at least one instance a dog in this laboratory has shown no alteration in the metabolism with a difference in the external environment of 10°C. Only with the onset of shivering does the metabolism increase perceptibly. It should be stated, however, that this investigation is by no means complete, and it is not the purpose here to enter into a discussion of the influence of temperature environment upon the heat production of lower animals. With men the evidence points strongly towards the constancy of metabolism irrespective of moderate changes in atmospheric environment. This has likewise been borne out by the results of experiments made by Schlossmann and Murschhauser<sup>27</sup> on sleeping infants.

*Diurnal variations in metabolism.*

The routine for conducting experiments for the determination of the basal metabolism usually involves a series of experiments in the early morning in the post-absorptive condition; that is, before food is taken. Very little data are available to show whether or not the course of the metabolism throughout the day is altered materially. The best evidence that the Nutrition Laboratory possesses is that obtained with the fasting subject, who, as has already been noted, had a considerably greater metabolism awake than asleep. Experiments made with this subject in the afternoon after a day spent in talking and various experimental tests, but with little muscular exercise, showed that he invariably had a higher basal metabolism than he did in the forenoon. While of course the metabolism of a subject living under these artificial conditions may not be compared with that

<sup>27</sup> A. Schlossmann and H. Murschhauser: *Biochem. Ztschr.*, xxxvii, p. 1, 1911.

of a normal individual, nevertheless it is a fact that this organism which, aside from the absence of food, was otherwise normal, had three sharply defined metabolic planes. These values were: first, a value when the subject was asleep in the bed calorimeter; second, a value obtained when he was awake lying on a mattress about 9 o'clock in the morning; and third, a value obtained under the same conditions in the late afternoon. Using as a basis the metabolism for the night during sleep, we find that the metabolism in the morning with the subject awake had increased 14 per cent, and in the late afternoon, under the same conditions, had increased 22 per cent. It is furthermore of interest to note that many of the infants studied by Benedict and Talbot exhibited differences in metabolic levels, as indicated by an increased pulse rate and an increased gaseous metabolism, even though in sound sleep and with no evidence on the kymograph record of muscular activity.

• *Stimulus as influenced by prolonged fasting.*

Further striking evidence of the probable effect of a decreased stimulus is found with the fasting subject, who showed a depression in the metabolism wholly out of proportion with the changes in the body weight or in the body surface as computed by the Meeh formula. The heat production per square meter of body surface as computed on the morning of the first fasting day, that is, about eighteen hours after the last food, was 859 calories. As the fast progressed, the heat production on this basis of computation fell until a minimum of 668 calories was observed on the morning of the twenty-third day of fasting. No disproportion between the body weight and body surface could be assumed with this individual corresponding to this difference in heat production, and we are again convinced of the fact that here we deal with a variation in intensity of a true stimulus to cellular activity. This is furthermore emphasized by the fact that after the twenty-third day there was a distinct tendency for the metabolism to rise which was accompanied by a measurable increase in the pulse rate. Thus the tendency to depress the metabolism, due to the continued loss of protoplasm by fasting, was actually overcome by the unknown stimu-

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lus increasing the cellular activity of the remaining body substance, ultimately resulting in a positive increase in the basal metabolism.

*Influence of character of diet.*

While it may seem questionable to introduce here a discussion of the influence of a carbohydrate-free diet upon the post-absorptive metabolism, it has been demonstrated that with the acidosis resulting from the ingestion of a carbohydrate-free diet, there is a distinct increase in the basal metabolism of normal individuals. This increase is clearly not due to a change in the body weight or the body surface or to variations in the mass of active protoplasmic tissue, but must be due to an alteration in the stimulus to cellular activity, the presence of acids in the body stimulating the cellular activity to a higher level.

*After-effects of severe muscular work.*

Finally, mention should be made of the marked after-effects of severe muscular work noted by Benedict and Cathcart<sup>28</sup> with their professional athlete, M. A. M., who had not partaken of food for nearly twenty hours, during which time he had performed an enormous amount of muscular work. After the cessation of work, the metabolism showed a prolonged though steadily decreasing influence of the preceding muscular activity. The investigators believe that this stimulus to the cellular activity continued for a long time after all external evidence of muscular activity had ceased. Indeed, so long did the stimulus continue that some writers might ascribe at least a part of the increased metabolism noted with athletes as compared with normal individuals to the possibility of the after-effects of the muscular activity on the preceding day. Of most importance, however, is the fact that as a result of excessive muscular activity the cellular activity may be increased enormously and maintained at a high level for a considerable period after the cessation of the work, thus clearly establishing a higher, though continually decreasing, metabolic plane.

<sup>28</sup> Benedict and Cathcart: *loc. cit.*

## SUMMARY.

It is thus obvious that in studying the basal metabolism of a normal individual, we have a much larger number of factors to deal with than has hitherto been recognized.

Unquestionably body weight plays an important part. In general, large bodies give off larger amounts of heat than smaller ones, but there is no direct relationship between the total body weight and the total heat production.

The mathematical relationship between the body surface and the body weight established by direct measurement has led to the general belief that the heat production is determined by the body surface, since an approximate relationship has been frequently noted between them. Careful analysis of metabolism measurements obtained on athletes, normal men and women, and normal and atrophic infants, leads to the conclusion that the metabolism or heat output of the human body, even at rest, does not depend upon Newton's law of cooling, and is, therefore, not proportional to the body surface. While certain disturbances in this supposed relationship between the heat production and the body surface may correctly be ascribed to errors in the formulae used for computing body surface, nevertheless the vast bulk of the evidence, particularly with athletes and with infants, and to a considerable extent with so called normal individuals, shows that the variations between metabolism and body surface are far outside of any possible errors in formulae.

Body composition, *i.e.*, the proportion of inert body fat and active protoplasmic tissue, has a great influence upon the basal metabolism. The tendency toward the greater metabolism shown by athletes in comparison with non-athletes may thus be explained by the greater muscular development as indicating a larger proportion of active protoplasmic tissue. The apparent influence of sex, as brought out in the comparison of the metabolism of men and women, may also be attributed to the greater proportion of inert body fat in the latter, with a consequent smaller amount of active protoplasmic tissue. It has also been seen that height is a factor in determining the basal metabolism, since in comparing individuals of like body weight and age, but widely varying height, the taller individual has usually the

greater metabolism; this is likewise due without doubt to the fact that the taller individual has the larger amount of active protoplasmic tissue. All these variables deal directly with the mass of the heat-producing organism; *i.e.*, the amount of active protoplasmic tissue.

We have still another very important factor; ~~namely~~, the stimulus to cellular activity. This stimulus is influenced by a number of factors. One of these factors is age, and it has been noted that with the growing organism of youth, there is a much greater cellular activity than with the adult, and a consequent higher metabolism. It has been brought out, however, that in old age there may be actual atrophy of protoplasmic material.

Sleep has also been shown to have an influence upon the basal metabolism, the stimulus to the cellular activity being greater with an individual when he is lying awake than when he is asleep.

Considerable fluctuations in the basal metabolism have been found from day to day not only with a fasting man but with normal individuals studied over considerable periods of time. These variations could not logically be attributed to changes in body weight or body surface, and obviously there was no change in height. Even in the course of twenty-four hours, the fasting subject was found to have three distinct metabolic planes, showing clearly a diurnal variation in the stimulus to the cellular activity.

Still other factors considered as influencing the stimulus to cellular activity are prolonged fasting, the character of the preceding diet, and the after-effects of severe muscular work.

From the evidence gathered with the various subjects studied, it is clear that the basal metabolism of an individual is a function, first, of the total mass of active protoplasmic tissue, and second, of the stimulus to cellular activity existing at the time the measurement of the metabolism is made. Apparently at present no law can be laid down that will cover both of these important variables in the basal metabolism of an individual.



## A RESPIRATION APPARATUS FOR SMALL ANIMALS.

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The increasing use of determinations of the respiratory exchange as an index of alterations both in the character and in the amount of the basal metabolism calls for an apparatus that may be satisfactorily used for small animals, with which the carbon dioxide output and the oxygen intake may be determined with sufficient accuracy to give reliable respiratory quotients. The determination of the carbon dioxide output is relatively simple with small animals, and a number of methods have been used to secure this end. Perhaps the simplest is the closed chamber respiration apparatus, in which the carbon dioxide is allowed to accumulate and samples of air are analyzed from time to time. Another method commonly used is to pass a current of outdoor air through the respiration chamber, measure the amount of air passing through, and note the increment in the carbon dioxide percentage.

On the other hand, the determination of the oxygen consumption of small animals presents technical difficulties so great that it is rarely attempted. In a relatively few experiments a tracheal cannula has been used for short periods of observation, the inspired air being separated from the expired air by a system of valves; the expired air may thus be measured, sampled, and analyzed. This method has recently been employed by Tangl,<sup>1</sup> but nearly all his animals weighed 5 kgm. or over. The indirect method of Haldane,<sup>2</sup> and more recently the combined direct and indirect method of Fredericia<sup>3</sup> have also been occasionally employed for the determination of the carbon dioxide output and the

<sup>1</sup> F. Tangl: *Biochem. Ztschr.*, xxxiv, p. 1, 1911.

<sup>2</sup> J. S. Haldane: *Jour. Physiol.*, xiii, p. 419, 1892.

<sup>3</sup> L. S. Fredericia: *Biochem. Ztschr.*, liv, p. 92, 1913.



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oxygen consumption of small animals; but the determination of these factors for animals as small as a rabbit or a guinea pig has not been common in laboratory technique.

Experiments with small animals are of value in the investigation of many problems and may be extended twenty-four hours or more, thus permitting the collection of appreciable amounts of carbon dioxide and the absorption of corresponding amounts of oxygen. Such long periods of observation will necessarily be accompanied by more or less muscular activity. It is becoming more and more evident to workers in metabolism that in studying basal values or the superimposition of a factor affecting metabolism upon the basal value, only periods of complete muscular repose may advantageously be used; on the other hand, there are numerous problems, particularly those in which the character of the katabolism is to be studied, which do not require absolute muscular repose for their investigation, although it is always desirable. For such experiments a respiration apparatus or, more properly, a modification of the universal respiration apparatus, has been used in this laboratory for the measurement of both the carbon dioxide production and the oxygen consumption of animals as small as a rabbit or a guinea pig. A description of this modified apparatus follows.

The universal respiration apparatus has been extensively employed in this laboratory for the past few years. It has been readily adapted to experiments with a man at work and has been successfully employed for such experiments when the carbon dioxide production was as great as 2500 cc. in one minute. It has also been applied to the measurement of the metabolism of infants. The universal respiration apparatus is now established in several other physiological laboratories. Thus far it has been used in these laboratories primarily for studies on men, and when such studies are not in progress, it has been unemployed. I purpose showing here how the apparatus may be so modified by a simple valve system that it may also be readily applied to the measurement of the metabolism of animals weighing but 1 or 2 kgm.

With the universal respiration apparatus,<sup>4</sup> as used most frequently for studying the respiratory exchange of man, the subject

<sup>4</sup> F. G. Benedict: *Deutsch. Arch. f. klin. Med.*, cvii, p. 156, 1912.

breathes through a nosepiece or a mouthpiece and draws air into the lungs from the ventilating air current. The displaced air ultimately passes through a carefully counterpoised spirometer which moves up and down with each respiration. By connecting a small respiration chamber to the ventilating air circuit and allowing the spirometer to act as a supplementary expansion chamber, air may be circulated through the respiration chamber at any desired rate of speed. An animal confined in the respiration chamber gives off carbon dioxide with each expi-

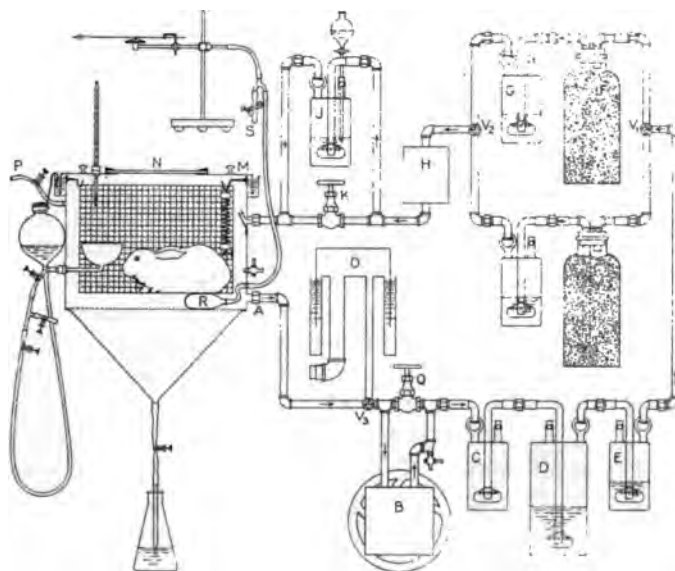


FIG. 1. Schematic outline of respiration apparatus for small animals.

ration, which is later removed from the ventilating air current; it also absorbs oxygen with each inspiration, this consumption of oxygen being indicated by the decreased volume of air in the spirometer; the loss of oxygen from the air is ultimately measured by noting the amount required to supply the deficiency. A diagram of the apparatus as used for experiments with small animals is given in Figure 1.

*General description of apparatus.* The course of the circulating air current is shown in the schematic outline of the absorbing

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apparatus at the right side of the figure. This does not differ in general from that illustrated in previous communications in which this form of apparatus has been described as used with man and with infants. The ventilating current is kept in motion by a rotary blower *B*, which is driven by an electric motor. The air leaves the chamber at the point *A* and, after passing through the blower, is forced first through an empty glass vessel *C*, which serves as a safety trap, and then through the two so called "Williams bottles" *D* and *E* containing sulphuric acid which removes the moisture. The air, which is now water-free but contains the carbon dioxide produced by the animal and also lacks the oxygen consumed, passes into the 2-way valve *V*<sub>1</sub>, where it may be deflected into the bottle *F* containing soda lime for the absorption of the carbon dioxide. Since the soda lime is necessarily somewhat moist, moisture is yielded to the air in its passage through the vessel *F*, which must be collected or otherwise it causes an error in the measurement of the carbon dioxide. This moisture is absorbed by the sulphuric acid in the accompanying bottle *G*. The air then passes through a second 2-way valve *V*<sub>2</sub> to a small brass can *H*, containing dry sodium bicarbonate, this salt removing the unweighable traces of acid vapor retained by the air as it passed through the vessel *G*. Sufficient moisture for comfortable breathing is supplied to the air in its passage through the water in the glass vessel *J*; the proportion of the air passing through the water in *J* is controlled by means of the by-pass valve *K*. The air is then returned to the respiration chamber. This chamber, which is cylindrical in form, is constructed of copper and has a cover *M* with a water seal. The glass plate *N* in the cover gives opportunity for observation of the animal.

*Spirometer.* As thus arranged the entire ventilating system and the chamber are of rigid wall construction and permit no automatic fluctuations of the volume such as would be caused by changes in barometric pressure or temperature. The spirometer of the universal respiration apparatus, which provides for such fluctuations in the volume of the air, is usually attached to the ventilating system near the pipe on the intake side of the blower, and in Figure 1 it is shown as attached to a 3-way valve at this point (*O*). Ordinarily the spirometer is so connected that the entire volume of the ventilating air current passes through the

spirometer, but in the adaptation of the apparatus for experiments with small animals, this is unnecessary. As the volume of air in the chamber changes by reason of changes in the barometer, in the temperature, or on account of the absorption of oxygen by the subject, the spirometer freely adapts itself to the variations in volume, so that the pressure inside the chamber is always the same as that of the atmosphere. From the readings of the height of the spirometer on an attached scale, a mathematical estimate of the variations in the volume may be readily obtained. Oxygen to replenish that used by the animal is added through the pipe *P* whenever such need is indicated by the height of the spirometer bell.

Thus the respiratory system of this apparatus consists of a chamber,<sup>5</sup> ventilating air current, absorbing vessels to remove the carbon dioxide and water, spirometer to provide for changes in the volume and for indicating the need of oxygen in the chamber, and a tube for the introduction of oxygen.

*Rate of ventilation.* The Crowell rotary blower, commonly supplied for the universal respiration apparatus, is capable of maintaining a ventilating air current of 100 liters per minute, such as would be used in experiments with severe work. In experiments with small animals, such a high rate of ventilation is obviously unnecessary. Indeed, the rate employed for respiration experiments with a resting man, *i.e.*, 35 liters per minute, is much too large. It has been found impracticable to reduce the speed of the blower so that it would deliver but 2 to 3 liters of air per minute, but the effective ventilation of the chamber may be reduced to any desired amount by means of the by-pass valve *Q*. When this valve is open, the air circulates only around the blower and is not forced through the ventilating system. By partially closing the valve any desired rate of ventilation may be

<sup>5</sup> All our observations on single rabbits and guinea pigs have been made with a chamber having a volume of 17 liters. Obviously the smallest volume consistent with the size of the animal is to be desired. By placing the water seal at the bottom and varying the size and shape of the covers, various heights and sizes of chambers may be constructed and readily attached to the absorbing apparatus. All pipes may advantageously remain in the bottom of the chamber. Internal supports of different heights for the various sizes of swinging cages present no difficulties to the mechanician.

readily obtained. The ordinary rate required for a rabbit weighing 1 or 2 kgm. would be approximately 2 liters per minute.

*Measurement of the carbon dioxide produced.* The amount of carbon dioxide produced is determined by weighing the soda lime bottle *F* and its accompanying sulphuric acid bottle *G* on a balance which is sensitive to 0.01 of a gram, the Sauter balance being found the most satisfactory for this purpose. At the end of any given experimental period, the air current may be deflected to a second set of carbon dioxide absorbers by turning the 2-way valves  $V_1$  and  $V_2$  simultaneously, and the experiment proceeds without interruption.

*Measurement of the oxygen consumption.* The amount of the oxygen consumed may be determined in a number of ways. The spirometer of the universal respiration apparatus has a flexible volume of not far from 2 to 3 liters. This spirometer can be filled with pure oxygen which may gradually be allowed to enter the system as needed or may be immediately introduced; for we have no evidence as yet that atmospheres with an oxygen percentage of 30 per cent or thereabouts alter the metabolism in any way. With this method, by simply noting the fall of the spirometer and making suitable corrections for changes in the barometer and in the temperature, the oxygen consumption is readily computed. This method has the disadvantage that the oxygen must be introduced from time to time; the length of the period that the apparatus may be left unattended is thus determined solely by the size of the spirometer. By attaching a large spirometer to the pipe *P* as, for example, a 50-liter Tissot or Bohr spirometer, the oxygen consumption may be determined in twenty-four hour periods and the apparatus allowed to operate continuously without attention for the entire twenty-four hours. With this method we have used a Tissot spirometer which is filled at the beginning of the experiment with pure oxygen.

Since the volume of air in the respiration apparatus is only 30 liters or less, it will be seen that if a spirometer containing 50 liters of pure oxygen were attached, there would be a rapid diffusion of the gas and the percentage of oxygen in the ventilating current would soon approximate 50 or 60 per cent. To prevent such diffusion, a special form of communication between the spirometer and the pipe *P* has been used, which is shown in Figure

2. For this a small 3-necked flask has been employed.<sup>6</sup> Through the middle opening (A) connection is made directly with the large spirometer; in one of the other openings a glass tube with stopcock (B) is inserted to be used for introducing the oxygen into the spirometer; in the third opening is a capillary glass siphon U-tube (C), 65 cm. long, which connects the supply of oxygen

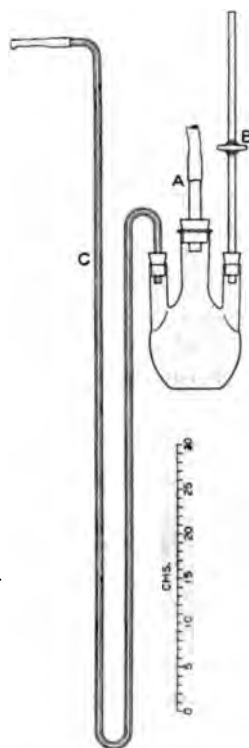


FIG. 2. Oxygen trap for connecting a large spirometer with the respiration apparatus.

with the respiration chamber. The oxygen, being heavier than the air in the respiration chamber, is trapped at the bottom of the long U-tube and thus is delivered into the chamber only as actually required. When the Tissot or Bohr spirometer is used, the

<sup>6</sup> Any other form of bottle with a 3-holed rubber stopper may be used equally well.

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3-way valve  $V_3$  (Figure 1) is turned so that no air passes into the spirometer  $O$ . Since this spirometer is the only flexible part of the ventilating apparatus, there will be a slight diminution of pressure inside the respiration chamber as the oxygen is absorbed. This diminution in pressure will then be immediately compensated by an inflow of oxygen from the large Tissot or Bohr spirometer through the capillary U-tube of the oxygen trap.

Proof of the efficacy of this method of introducing oxygen may be seen in the details of an experiment reported subsequently, showing that after a three day experiment with a rabbit the proportion of oxygen in the air inside the chamber was 20.13 per cent. It is clear, therefore, that under these conditions there is no appreciable diffusion between the normal air in the chamber and the pure oxygen in the spirometer. By noting the differences in level of the Tissot or Bohr spirometer, and correcting for the changes in temperature of the spirometer bell and the changes in the barometer, the oxygen consumption may be readily computed.

Another method of introducing oxygen into the ventilating system may be that employed with the large respiration calorimeters in this laboratory;<sup>7</sup> namely, by means of a valve which is electrically actuated and connected with the reduction valve on a small cylinder of oxygen. Oxygen may thus be automatically introduced into the chamber as the bell of the small spirometer  $O$  falls. By weighing the oxygen cylinder before and after the experimental period, the amount of oxygen admitted may be measured.

Finally oxygen may be periodically introduced by allowing the gas to flow from a cylinder of oxygen through a carefully calibrated gas meter,<sup>8</sup> such as is commonly used with the universal respiration apparatus.

For our experiments with small animals, we have found the method employing the Tissot or Bohr spirometer to be the most satisfactory. It is obvious that any type of accurate and readily calibrated spirometer may be substituted for either of these spirometers; in fact, the compensating devices of both the Tissot

<sup>7</sup> See F. G. Benedict and T. M. Carpenter: *Carnegie Institution of Washington Publications*, No. 123, Fig. 31, p. 68, 1910.

<sup>8</sup> Benedict: *Deutsch. Arch. f. klin. Med.*, cvii, p. 181, 1912.

and Bohr spirometers have no significance in this type of experiment, as an uncompensated bell may be used with equal accuracy.

*Graphic record of muscular activity.* The basal importance of knowing the muscular activity of even small animals during respiration experiments has hitherto been almost completely disregarded. Experience in this laboratory has shown that an intelligent comparison of experimental periods may not be made without some graphic indication of the variations in the muscular activity, and as far as possible it has been our custom to use for this purpose only periods of complete muscular repose. While ocular observations of the variations in muscular activity are worthless, fortunately the graphic registration of the degree of muscular repose is a simple matter. As may be seen in Figure 1, the animal is confined in a wire cage suspended by a stout spiral spring on one side and by a hook on the other. Under this cage is a soft rubber bulb (*R*) which is connected through the wall of the respiration chamber with a tambour and kymograph. Each change in the center of gravity of the animal's body produces a variation in the tension of the air in the bulb; this immediately affects the diaphragm of the tambour, whose writing point records the movement upon the kymograph. A test of the system of recording the muscular activity may be made by gently blowing into one part of the tube *S* and noting if the tension of the tambour rubber remains constant. By occasionally opening the tube at *S*, permanent distension of the tambour rubber by a major change in the position of the animal may be compensated. These graphic records have proved of great value in interpreting the results of experiments.

*Method of administering food and drink.* In experiments continuing several days, it is important to supply water and often food to the animal. In many laboratories it is customary to open the cage for one hour or less for this purpose and make observations of the metabolism only during the remaining twenty-three hours; such a method may be employed with this apparatus. When continuous experiments are preferred, the method of supplying water shown in Figure 1 has been found useful. If by means of this device the animal is given a milk diet, such as that employed by Laqueur,<sup>9</sup> experiments with this respiration apparatus

<sup>9</sup> E. Laqueur: *Ztschr. f. physiol. Chem.*, lxxxiv, p. 109, 1913.



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may be continued without difficulty for several days, if not weeks. The feces pass through a large mesh in the bottom of the cage, but are retained by a finer mesh. The urine passes through the meshes to the conical section in the bottom of the chamber and may be readily withdrawn.

The details of a typical experiment with a single rabbit, weighing 1600 grams, are given in Table I. On the first day of this experiment the rabbit was given 300 grams of carrots, and the muscular activity incidental to eating the food resulted in a higher metabolism; the respiratory quotient of 0.90 is clearly indicative of a predominating carbohydrate metabolism. On the subsequent days food was withheld; there was then a continual decrease

TABLE I.  
*Metabolism measurements with a rabbit (1600 gm.).*  
*(No food after first day.)*

DAY	CARBON DIOXIDE PRODUCED PER 24 HRS.	OXYGEN CONSUMED PER 24 HRS.	RESPIRATORY QUOTIENT
	<i>liters</i>	<i>liters</i>	
1st.....	28.99	29.87	0.90
2d.....	18.90	25.85	0.73
3d.....	16.63	22.32	0.75
4th.....	15.75	21.52	0.73
5th.....	14.61	20.38	0.72

in the oxygen consumption and carbon dioxide production, this being in full conformity with the general picture of the kymograph curves showing the muscular activity of the animal on these days; unfortunately the reproduction of these particular twenty-four hour kymograph curves is not feasible. The respiratory quotient for the last four days during the fasting period is indicative of a predominating fat combustion.

While the apparatus as previously described is primarily designed for long experimental periods, a special test was made to see what degree of success would be secured with an experiment on a much smaller animal and with a shorter experimental period. The results are given in Table II. In this test a single guinea pig, weighing 400 grams, was used. Instead of the large Tissot spirometer the small 2.5 liter spirometer, which is an integral



FIG. 3. Kymograph records showing muscular activity of a single guinea pig in three seven hour experiments.

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part of the unit respiration apparatus, was employed. Oxygen was admitted from a previously weighed cylinder of oxygen, the spirometer being filled and the apparatus allowed to run unattended until the spirometer became nearly empty; thus oxygen was admitted periodically. The experimental periods were five to seven hours long.

On the first day of the experiment the guinea pig ate a very large amount of carrots, and the respiratory quotient, 0.94, indicates a predominating carbohydrate combustion. On the second day no food was given and the quotient was 0.83, with a very marked decrease in the total metabolism as measured by the carbon dioxide production and oxygen consumption. On the third day the animal was still starving, but another variant was introduced in that the temperature of the chamber was decreased

TABLE II.  
*Metabolism measurements with a single guinea pig (400 gm.).*  
(No food after first experiment.)

EXPERI- MENT NO.	PERIOD	CARBON DIOXIDE PRODUCED		OXYGEN CONSUMED		RESPIRA- TORY QUOTIENT
		Total	Per hr.	Total	Per hr.	
		<i>liters</i>	<i>cc.</i>	<i>liters</i>	<i>cc.</i>	
36	9.36 a.m. 4.37 p.m.	3.02	430	3.21	457	0.94
37	11.37 a.m. 4.59 p.m.	1.45	270	1.75	326	0.83
38	9.35½ a.m. 4.16 p.m.	1.91	286	2.53	379	0.75

from 23.4°C. to 17.3°C. Under these conditions the animal was very much more restless and we note in the results a decreasing respiratory quotient, indicating a predominating fat combustion and an increase in the carbon dioxide production and oxygen consumption due to the activity. The kymograph records for the three seven hour experiments are reproduced herewith (Figure 3) and show clearly the influence of muscular activity upon the metabolism and the consequent importance of knowing the muscular activity when interpreting the metabolism measurements.

It is not the purpose of this paper to discuss the metabolism of a rabbit or a guinea pig in the early stages of fasting or as influenced by cold or muscular activity, these experiments being here reported only as typical cases showing primarily the flexibility of the apparatus.

*Metabolism in artificial atmospheres.* An apparatus of the closed circuit type, such as this, is readily used for studies of the metabolism of small animals in artificial atmospheres, and an extended research upon the metabolism and pathologic changes in small animals while breathing oxygen-rich gaseous mixtures is now in progress in this laboratory. In this type of experiment the animal remains in an atmosphere containing 90 to 95 per cent of oxygen for several days continuously. Under these conditions, the carbon dioxide production and oxygen intake have been satisfactorily determined.

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## SIMPLE QUARTZ MERCURY-VAPOR LAMPS FOR BIOLOGICAL AND PHOTOCHEMICAL INVESTIGATIONS.

By W. T. BOVIE.

*(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)*

(Received for publication, January 18, 1915.)

The quartz mercury-vapor lamps described below were made by the writer for use in connection with some experiments on the biological and chemical effects of ultraviolet light. At the time the lamps were made there were no quartz mercury-vapor lamps on the market in this country. The lamps gave, and are still giving, after four years' use, excellent results. They are less expensive than any of the commercial lamps known to the writer; and, furthermore, as will be seen, the design of these "home-made" lamps can be altered to suit particular requirements.

These lamps are not difficult to make. The quartz must be worked in an oxyhydrogen flame, and the burner must be adjusted so as to give the highest temperature possible; but the technique required for shaping the lamps is simpler than that required to make an ordinary T-joint in glass tubing, since the quartz joints do not have to be annealed. A good vacuum pump is required for exhausting the lamp. The greatest difficulty is found in making the seals at the points where the electrodes enter. The literature contains a number of more or less complicated and impractical methods, but the seals described below are easily made and are efficient.

It may be cheaper at present to have all the quartz parts of the lamp made at the factory. As stated above, these lamps were made before quartz tubing was being manufactured in this country.

Figure 1 shows a simple form which the lamp may take.<sup>1</sup> A is a quartz tube 1 cm. in diameter. One end is bent downward

<sup>1</sup> The form of this lamp is copied from a lamp made by Dr. C. A. Kraus.



to form the positive electrode *C*. A quartz tube of the same diameter is sealed in, near the other end, to form the negative electrode *B*. The distance between *B* and *C* is about 10 cm. The end of the tube *A* forms a condenser. The lamp is sealed, after exhausting at *H*. The tube *A* should be inclined to the horizontal position at an angle of  $6^\circ$  to  $7^\circ$ , the end *J* being higher, so that the condensed mercury will flow back into *C*. Pieces of 5 mm. quartz tubing are sealed to the lower ends of *B* and *C*. These

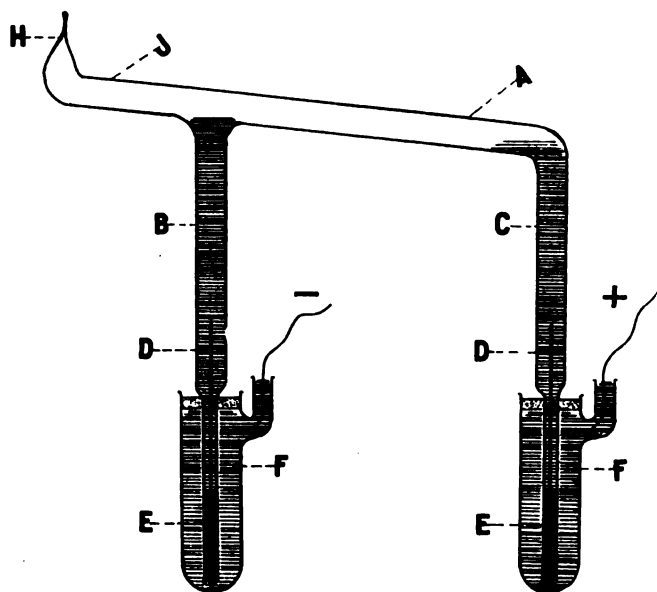


FIG. 1.

in turn are set in mercury cisterns *F*, *F* (made of glass; test-tubes may be used). The small tubes are sealed at *E* with De Khotinsky cement.<sup>2</sup> The detail of the seal is shown in Figure 2. The bulb of wax on the outside is necessary in order to make the seal air-tight. The iron wire *D* conducts the electric current through the wax seal. The mercury cisterns *F*, *F* radiate heat and also make the seal at *E* more secure by furnishing an additional mercury seal.

<sup>2</sup> This cement may be obtained from A. De Khotinsky, 6026 Drexel Ave., Chicago, Ill.

During the process of pumping the lamp should be turned, to allow the mercury to flow out of *B* and *C*, so as to free any imprisoned air bubbles. The lamp should then be placed in an upright position and the mercury in the upper parts of *B* and *C* boiled vigorously, keeping the wax at *E* cool by having the lamp stand in a dish of water. Lastly, the lamp should be connected with a 110 volt direct current (with suitable resistance in series with it), and run for one or two hours. When it appears to run evenly the vacuum pump should be stopped, and the lamp sealed off at *H*. The tube *A* should be drawn down to a small diameter at this point before the pumping begins.

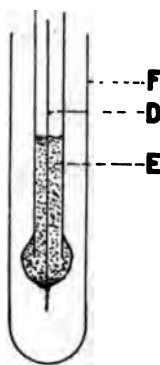


FIG. 2.

The lamp should be used on a 110 volt direct current with 5 or 6 sixteen candle power bulbs in multiple as resistance. The lamp will carry from 1.25 to 1.5 amperes, depending upon the amount of cooling at the electrodes *B* and *C*. When convenient it is desirable to arrange the experiments so that the lamp can stand in a dish of water, with the water surface well up on *B* and *C*. The lamp is started by tipping, or a Bunsen flame may be directed upon the surface of the electrode *C*. When the mercury boils the arc will start. As the lamp is made of quartz there need be no fear of breaking by sudden heating or cooling.

The tube *A* between *B* and *C* should be made of transparent quartz, but all other parts can be made of the cheaper translucent, fused silica. A lamp made for physical experiments, where only a small beam of ultraviolet light was needed, was made

entirely of fused silica, save for a small space between *B* and *C*, about 1 cm. long, where a small piece of transparent quartz was inserted.

Figure 3 shows a convenient arrangement for an experiment in photochemistry. The drawing shows a cross-section of the lamp, baths, tubes, etc., as used by the writer in studying the temperature coefficient of the coagulation of egg-albumin by ultraviolet light. *A, A* are copper trays set in the insulating case *B*. *C* is the lamp. The quartz test-tubes which hold the albumin are supported at *D* by the shelves *E, E*. The water in one of the trays is heated by the electric heating coil *F*, while the water in

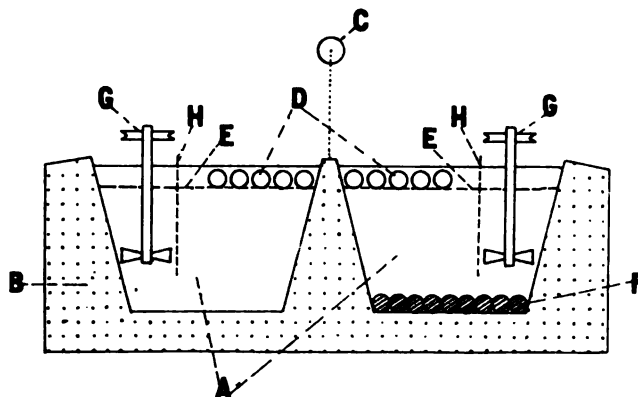


FIG. 3.

the other tray is kept at  $0^{\circ}$ , by placing chopped ice below the shelf *E*. The electric current in the heating coil is controlled by a thermoregulator. *G, G* are stirrers. The partitions *H, H* serve to prevent the stirrers from disturbing the surface of the water, and thus causing irregular variations in the reflection of light at the surface of the water. In this experiment the mercury cisterns (shown at *F, F* in Figure 1) stood in the ice water bath.

Figure 4 shows another of the possible forms of the lamp. It will be seen that the tube *A* is vertical. The electrode *C*, which is left open at the bottom, dips into a cistern of mercury *M*. This lamp was designed to run on a 220 volt circuit, and the distance between *B* and *C* is 30 cm. The lamp, however, will work as well on a 110 volt circuit; for, as will be seen presently, the

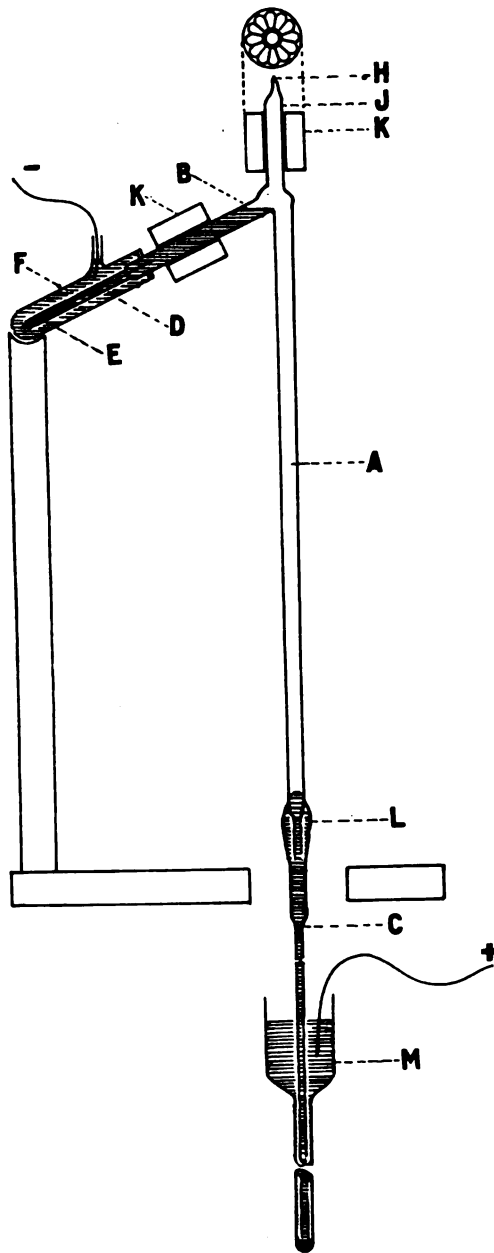


FIG. 4.

distance between *C* and *B* is easily adjusted. The arc is started in this lamp by elevating the mercury cistern *M* until the mercury in *C* comes in contact with that in *B*. The distance between the surface of the mercury in *M* and the surface in *C* is equal to the height of the barometric column, since the surface at *M* is exposed to the air. When the lamp is running on a 110 volt circuit the arc is not stretched down as far as when running on 220 volts. In place of the mercury cistern *M*, one can use a smaller chamber closed at the top, with the air space above the mercury connected with a water aspirator. The height of the mercury in *C* can then be controlled by varying the air pressure above the mercury in the cistern. With this arrangement the distance between *M* and *C* may be shorter. An air trap *L* is inserted between *M* and *C* to prevent the accidental entrance of air bubbles into *A*.

It will be noticed that the condenser *J* is not in a line with *A*, but is a little to one side. This is important, for a certain amount (about half) of the condensed mercury must be returned to the electrode *B*, else it will boil dry. The condenser *J* and the electrode *B* are provided with copper radiators, *K*. (The radiators were taken from discarded Nernst lamps.) By using these radiators the lamp could be made to carry a current of four amperes. With this current density and with an arc 30 cm. long the lamp emits such an abundance of ultraviolet light that the air in the room is quickly filled with ozone. The photochemical power of the lamp may perhaps best be judged from the fact that an exposure of one or two minutes at a distance of one meter will produce such a "sunburn" as to cause the skin to peel. Those who undertake work with quartz mercury-vapor lamps are warned *never to look at them while they are running*, except through a screen of red glass. An exposure of a very few seconds is sufficient to cause most painful consequences. The pain does not follow immediately after the exposure, but there is a latent period of several hours, so that one is not aware of the injury at the time of the exposure.

## THE METABOLIC RELATIONSHIP OF THE PROTEINS TO GLUCOSE.

By N. W. JANNEY.

(From the Chemical Laboratory of the Montefiore Home, New York.)

(Received for publication, January 28, 1915.)

The importance of glucose as an intermediary metabolic product is constantly becoming more evident. Through the work of Knopf, Glässner and Pick, Embden, Lusk, Ringer and Dakin,<sup>1</sup> in which depancreatized or phlorhizinized dogs were used, it has been shown that the majority of the amino-acids occurring in considerable amounts in the protein molecule are convertible into glucose.

The fact that various proteins yield large amounts of sugar in the diabetic phlorhizinized animal has been fully established. As the amino-acids, rather than higher complexes, have been shown in recent years to be the most important direct products of protein digestion, it seems reasonable that glucose derivable from catabolizing proteins is formed from the glucogenetic amino-acid complexes present in any particular protein. These sugar-yielding amino-acids vary markedly in amount in different proteins. It follows, therefore, that the proteins in their catabolism may be found to yield amounts of sugar proportional to the amount of glucogenetic amino-acid radicals present in them.

Experiments with this end in view have yielded results which may thus be summarized. *Each protein produces a definite amount of glucose in the phlorhizinized organism. The various yields represent 50 to 80 per cent by weight of the protein administered. These yields approximate the ratios which the glucogenetic amino-acids of the proteins in each case bear to the total amino-acids, as actually determined by hydrolysis.*

<sup>1</sup> H. D. Dakin: this *Journal*, xiv, p. 321, 1913. For the literature quoted in this article, when not especially noted, reference may be made to the comprehensive monograph of Lusk: Phlorhizinglukosurie, *Ergebn. d. Physiol.*, xii, p. 315, 1912.

## 322 Metabolic Relationship of Proteins to Glucose

*Material.* Pure proteins were used in the experiments. Calculations throughout the paper have been based on anhydrous material. Water determinations made by drying in vacuum, usually at 110°, have for the sake of brevity been omitted.

*Casein.* Prepared according to Hammarsten.

0.1781 gm. gave 0.0278 gm. nitrogen.....N=15.6 per cent.

According to Hammarsten.....N=15.6 per cent.

*Ovalbumin.* Prepared from Merck and Co's. "soluble egg albumin," preparations of which were not entirely soluble in water. They contained globulins and ovomucoid. This material is, however, somewhat more convenient to use than egg-white. Even with the help of several existing descriptions, technical difficulties in the preparation of pure ovalbumin may still be met. A mode of preparation is therefore given here.

*Preparation of ovalbumin.* From a dilute unfiltered solution of Merck's soluble egg albumin or the diluted egg-white, the globulins and ovomucin (Eichholz) are removed by adding an equal volume of saturated ammonium sulphate solution. The albumin is obtained from the filtrate by dissolving in it powdered solid ammonium sulphate until a further addition to a filtered sample causes no precipitation. Filter off and dissolve in water. Use preferably distilled water throughout. Reprecipitate with ammonium sulphate in like manner and repeat this process twice. Collect the albumin on a folded filter, wash with saturated ammonium sulphate solution, suspend the finely divided precipitate in a small volume of water, and dialyze for three days to free from the major portion of the sulphate. The albumin goes into solution. It may be coagulated by pouring in a fine stream into boiling water or by immersing the flask containing it in boiling water. The coagulum rubbed fine is washed free from sulphate and ovomucoid by decantation or on large folded filters. Ovomucoid is tested for by evaporating the filtrate to very small bulk and adding four times as much 95 per cent or absolute alcohol. A light flocculent precipitate denotes the presence of ovomucoid. The pure albumin is dehydrated by letting it stand in a finely divided condition for twenty-four hours or longer, under 95 per cent alcohol, and then washed with absolute alcohol and dry ether on a Buchner funnel, with a hardened filter. Damp air should be excluded during this process. The product, a snow-white powder, represents the total ovalbumin (conalbumin and egg albumin proper).

0.3652 gm. prepared from Merck's "soluble egg albumin" gave

0.05677 gm. nitrogen.....N=15.55 per cent.

0.2005 gm. prepared from egg-white gave

0.03184 gm. nitrogen.....N=15.8 per cent.

According to Osborne and Harris,

crystallized egg albumin.....N=15.5 per cent.

conalbumin.....N=16.1 per cent.

*Human serum albumin.* Prepared from ascitic fluid from cases of hepatic cirrhosis or cardiac insufficiency, according to a method similar to the one immediately preceding. Fine white powder. No analyses of human serum albumin seem to be reported. The nitrogen values differ slightly from that of horse serum albumin.

1. 0.3484 gm. gave 0.05481 gm. nitrogen. . . . . N = 15.7 per cent.
2. 0.3192 gm. gave 0.05033 gm. nitrogen. . . . . N = 15.8 per cent.
3. 0.2835 gm. gave 0.04458 gm. nitrogen. . . . . N = 15.7 per cent.

The analyses were made from different preparations. The material for analyses 1 and 2 was three times, that for analysis 3, six times precipitated with ammonium sulphate.

*Gelatin.* A German preparation ("Gold seal" WH No. 1866).

- 0.2994 gm. yielded 0.05186 gm. nitrogen. . . . . N = 17.3 per cent.

It was further purified according to the following method: A 20 per cent solution in warm water was allowed to solidify, the jelly-like mass finely divided by passing through a small meshed sieve, and for a period of two weeks washed at a low temperature with distilled water containing ether, which was changed daily. The material was then treated successively with 50 per cent, 95 per cent, and absolute alcohol and ether.

- 0.2867 gm. yielded 0.05076 gm. nitrogen. . . . . N = 17.7 per cent.

According to Hausmann. . . . . N = 18.0 per cent.

*Fibrin.* A finely pulverized preparation obtained from blood.

- 0.3965 gm. gave 0.06577 gm. nitrogen. . . . . N = 16.6 per cent.

According to Samuely. . . . . N = 16.9 per cent.

The vegetable proteins were prepared according to Osborne.<sup>2</sup>

*Edestin.* Prepared from hemp seeds.

- 0.2718 gm. gave 0.05115 gm. nitrogen. . . . . N = 18.8 per cent.

According to Osborne. . . . . N = 18.6 per cent.

*Gliadin.* Prepared from wheat flour.

- 0.2520 gm. gave 0.04429 gm. nitrogen. . . . . N = 17.6 per cent.

According to Osborne. . . . . N = 17.7 per cent.

*Zein.* Prepared from corn gluten meal.

- 0.3133 gm. gave 0.0506 gm. nitrogen. . . . . N = 16.2 per cent.

According to Osborne and Harris. . . . . N = 16.1 per cent.

*Mode of feeding.* Casein, ovalbumin, serum albumin, fibrin, and edestin were thoroughly moistened with water, and when not voluntarily eaten were fed through the stomach tube. Gelatin was prepared for administration by dissolving in about five times its weight of warm water. The jelly formed on cooling was finely divided and then fed, or the gelatin solution at 40° was introduced into the stomach by the stomach tube.

Gliadin when moistened with water or saliva becomes a dense glue-

<sup>2</sup> T. B. Osborne: Darstellung der Proteine der Pflanzenwelt, in E. Abderhalden: *Handbuch der biochemischen Arbeitsmethoden*, ii, p. 270, 1910.



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like mass, exceedingly difficult to handle or to swallow. A special feeding method was therefore devised as follows: The gliadin was dissolved in as small an amount as possible of  $\frac{N}{40}$  sodium hydroxide, and the solution was introduced into the stomach by the stomach tube, followed immediately thereafter by an equivalent amount of  $\frac{N}{40}$  HCl. The gliadin was thus deposited in a very soft digestible condition in the stomach.

### *Alkali-acid control experiment.*

	PERIODS		NITROGEN	GLUCOSE	G:N
	dy.	hrs.	gm.	gm.	
Weight of dog, 9.2 kgm. 384 cc. $\frac{N}{40}$ NaOH followed by 384 cc. $\frac{N}{40}$ HCl (80 cc. fluid per kgm.) by stomach tube given on 2d day.	1	12	3.77	13.11	3.48
	1	12	3.35	11.76	3.61
	2	24	7.44	26.09	3.50

The technique employed in this experiment was precisely the same as in the feeding experiments. Neither digestive disturbances nor influence on the glucose excretion was observed. The similarity of the gliadin partition curve with that of the proteins fed without the use of acid and alkali demonstrates also the reliability of this method of feeding. Very large amounts of less dilute alkalis and acids fed in this manner have, however, been shown by other experiments to give rise to diarrhea, diuresis, and even to metabolic disturbances giving rise to an increased glucose excretion.

Zein, on account of its hard consistence, is difficult of digestion and absorption, as is well known. Thus, in a recent paper of Osborne and Mendel,<sup>3</sup> reporting a comparative series of feeding experiments with proteins, the utilization of zein (average of seven experiments) was 65 per cent for the rat. This is considerably less than in the case of other proteins. For this reason a special method of preparing the zein for feeding was devised as follows:

Finely divided zein is shaken with sufficient  $\frac{N}{40}$  sodium hydroxide at room temperature to obtain a 5 per cent solution. This solution, which must not be allowed to stand longer than twenty-four hours, is precipitated by adding, while stirring, a like volume of  $\frac{N}{40}$  hydrochloric acid in a fine stream. The soft mass of zein is freed from excess of the neutral fluid by draining with suction on a Buchner funnel and then given to the animal through the stomach tube immediately after adding sufficient water to make up the standard amount required for the experiment. The filtrate was evaporated to dryness at 40°. The residue containing zein was then treated with absolute alcohol to take up the protein, the solution filtered and evaporated to dryness again at a low temperature, and finally treated with water to free it from traces of sodium chloride. The pure zein thus

<sup>3</sup> T. B. Osborne and L. B. Mendel: this *Journal*, xviii, p. 177, 1914.

obtained on drying usually amounted to about 0.15 gm. This amount was deducted from that weighed off for feeding. By the use of this method, as the protocols show, zein can be fed with certainty of obtaining as complete absorption as in the case of any other protein of this series.

A standard amount of protein containing usually 0.3 or 0.5 gm. nitrogen per kgm. was fed, at times with the help of a little dilute extract of beef. Extract of beef has been shown by Lusk not to influence the glucose elimination.

In view of the necessary employment of the stomach tube for feeding in many cases, the question of psychic glycosuria with consequent influence on the sugar excretion may be raised. By comparing the results, however, of experiments where the protein was eaten voluntarily with those where the tube had to be employed, we have never in a considerable experience seen results where a possible extra sugar excretion due to struggling could be detected. Hirsch and Reinbach<sup>4</sup> report that even continued and violent excitement in dogs is followed by but a relatively small increase of blood sugar. Even in this case the urine contained no glucose.

*Methods.* Two days previous to urine collection fasting began, and 1 gm. of phlorhizin was administered according to Coolen daily, in some cases every twelve hours. Periods of urine collection were separated by catheterization and washing out the bladder with aseptic precautions. The last washing terminated precisely with the period's end. Nitrogen was determined according to Kjeldahl, glucose according to the Pavy-Fehling method, and a polariscopic control was made in all cases. In certain of the casein experiments glucose was titrated according to Benedict.<sup>5</sup>

Much more care is required in the choice of dogs for the experiments here reported than is necessary for those aiming merely to demonstrate qualitatively a rise in the sugar elimination after ingestion of a given substance. Only hardy mature animals, preferably about two years old, whose general tone remains excellent during the entire experimental period, can be relied upon for accurate results. A weakly or young dog may excrete but 75 or even 50 per cent of the *maximal* yield of glucose obtainable from a given protein. In properly selected animals, however, if due care was observed, a surprising regularity in the amount of the glucose yields of different experiments was noted.

The amounts of protein fed were so chosen that the glucose yield even in larger dogs fell below 25 gm., with but one exception. Phlorhizinized

<sup>4</sup> E. Hirsch and H. Reinbach: *Ztschr. f. physiol. Chem.*, xci, p. 292, 1914.

<sup>5</sup> For these experiments and for a preparation of gliadin and of edestin, I am indebted to Dr. Isidor Greenwald, now of the Harriman Research Laboratory, New York.

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dogs excrete quantitatively glucose ingested in amounts at least up to 50 gm. or more.<sup>6</sup> For the calculation of the extra glucose the method of Lusk was adhered to. Though wide differences in the amounts of body protein spared by the ingested proteins were observed, they influenced but slightly the calculation of the extra glucose. Only one experiment with a very irregular nitrogen excretion did not lend itself to Lusk's method of calculation.

### PART I. THE RATE OF METABOLISM OF PROTEINS.

In the present study it is of fundamental importance that the proteins be administered in such a way as to insure maximal absorption and prompt elimination of their metabolic products. If these conditions are not secured the results obtained would have but little value. Mendel and coworkers<sup>7</sup> have carried out recent studies in this direction, the general conclusion reached being as follows: it is probable that the digestive products of *all* proteins are absorbed and eliminated at like speed provided that certain factors are allowed for. The latter are:

(1) Mechanical. A very finely divided, thoroughly softened, and hydrated protein in a pure state is digested and absorbed with great rapidity. Admixture of indigestible substances, such as cellulose, delays these processes in direct proportion to the relative quantity present.

(2) The amount of carbohydrate and fat as protein spacers, in the diet.

(3) Water intake with the meal.<sup>8</sup> The differences of previously reported experiments dealing with absorption and utilization of proteins can be accounted for on these grounds. With proper allowance for the factors mentioned, rapid and complete or nearly complete absorption of most proteins can be obtained.

<sup>6</sup> A. I. Ringer: *Proc. Soc. Exper. Biol. and Med.*, ix, p. 52, 1911-12; this *Journal*, xii, p. 431, 1912. See also Lusk: *loc. cit.*

<sup>7</sup> For the literature referred to in this section, reference may be made, when not especially noted, to E. P. Cathcart: *The Physiology of Protein Metabolism*, New York, 1912. L. B. Mendel and M. S. Fine: this *Journal*, x, pp. 303, 339, 345, 1911-12; xi, pp. 1 and 5, 1912. L. B. Mendel and R. C. Lewis: *idem.*, xvi, pp. 19, 37, 55, 1913-14.

<sup>8</sup> This is important as it has recently been demonstrated that water is an active gastric stimulant (O. Bergeim, M. E. Rehfuess, and P. B. Hawk: this *Journal*, xix, p. 345, 1914).

In the following experiments these studies have been extended to the phlorhizinized dog. Proteins were fed and hourly determinations of the glucose and nitrogen made. With a view of obtaining complete absorption, all the determining factors as above mentioned are given, it is thought, due consideration. In addition, it seemed wise to employ fasting animals as well as smaller amounts of proteins for feeding than have been generally employed hitherto. This latter was done because the writer has observed that the amounts of protein food large enough to impede complete absorption are much smaller than is generally supposed. No feces were passed in any of the experiments.

The curves show the percentage of the extra nitrogen and sugar excreted per hour. In each case the mean hourly nitrogen and glucose excretion was obtained from the fore- and after-periods immediately adjoining the nine hourly periods. The excretion in excess of this mean for these periods was then noted, and the sum taken as 100. The glucose and nitrogen curves for one protein show no extraordinary deviation from those obtained for the others. In each case the glucose excretion reaches its maximum at the second to the third hour, while the highest hourly excretion of the nitrogen is usually attained a little later, from the third to the fifth hour. Elimination of the glucose and nitrogen is complete by the ninth hour in all cases. An identically planned partition experiment was also carried out in the case of pure *zein*. As the experiment was imperfect it is not reported in detail. The glucose was completely eliminated by the ninth hour, the resulting curve being practically identical with those described in this article. The glucose and nitrogen curves<sup>9</sup> in the case of serum albumin are more flattened than any of the others. The probable explanation for this lies in the fact that in this experiment but little water was given with the protein (page 326). The two casein experiments are of interest as their nitrogen curves present three waves. A similar wave-like nitrogen excretion has been reported by others.<sup>10</sup> An entirely satisfactory explanation of this seems lacking.

<sup>9</sup> The author is indebted to Mr. Frank C. Csonka for this experiment.

<sup>10</sup> B. Tschlenoff: *Centralbl. f. Physiol.*, x, p. 177, 1896-97. O. Veraguth: *Jour. Physiol.*, xxi, p. 112, 1897. E. Haas: *Biochem. Ztschr.*, xii, p. 203, 1908.

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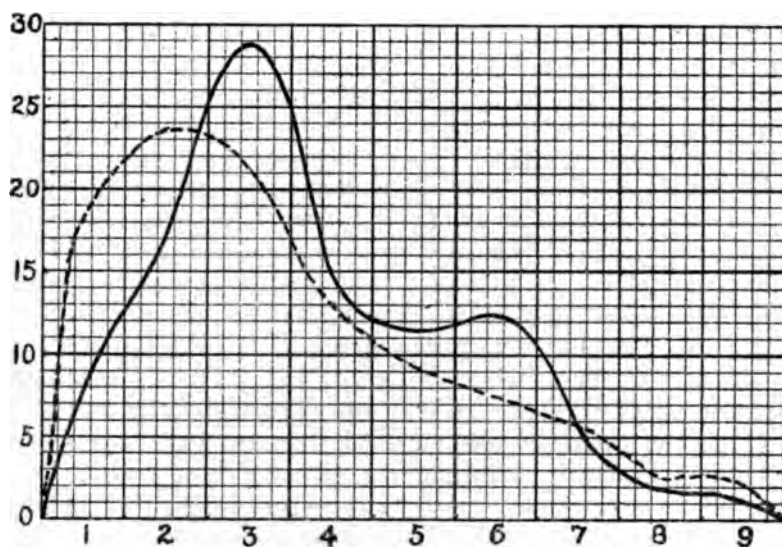


CHART 1. Casein I. In the curves height represents glucose and nitrogen hourly percentage excretion; length, hourly periods. The dotted curve represents glucose excretion, and the solid curve the nitrogen.

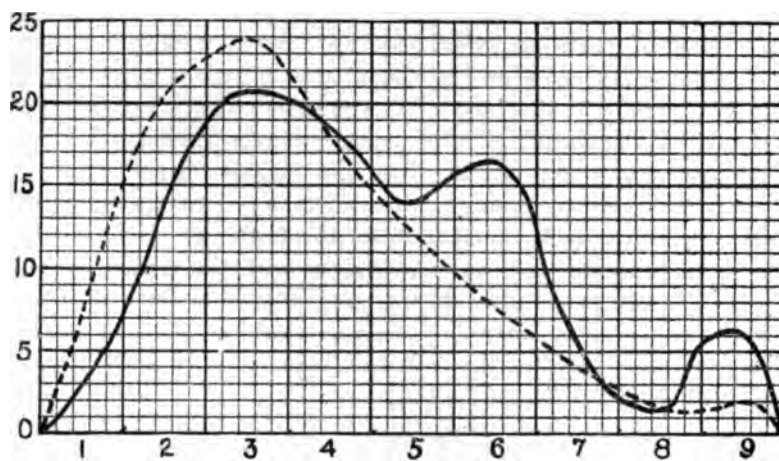


CHART 2. Casein II.

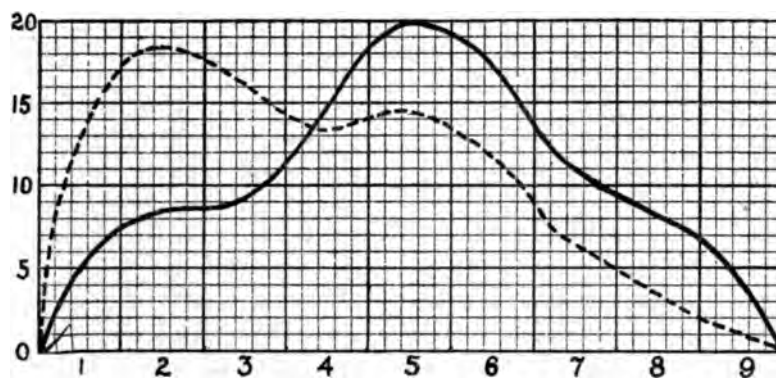


CHART 3. Serum albumin.

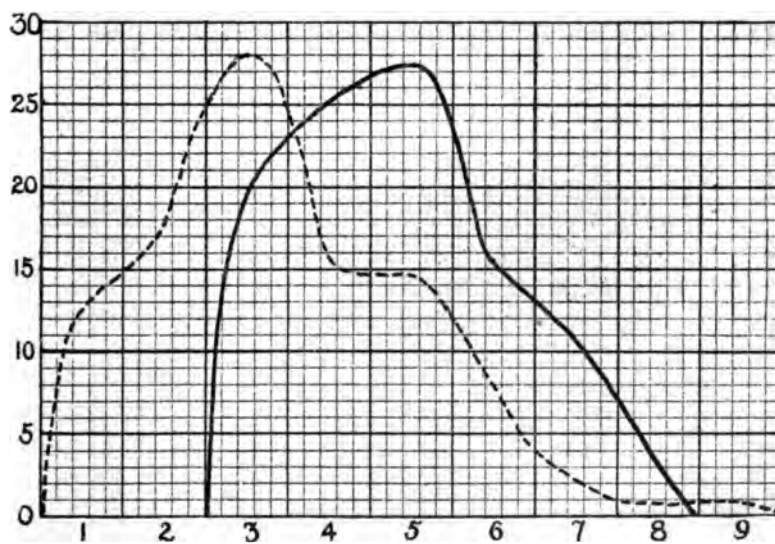


CHART 4. Gliadin.

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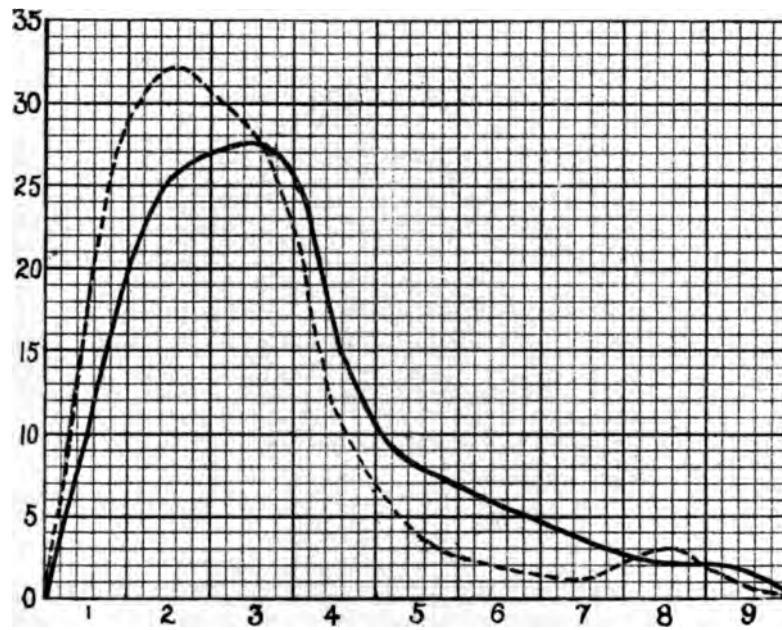


CHART 5. Edestin.

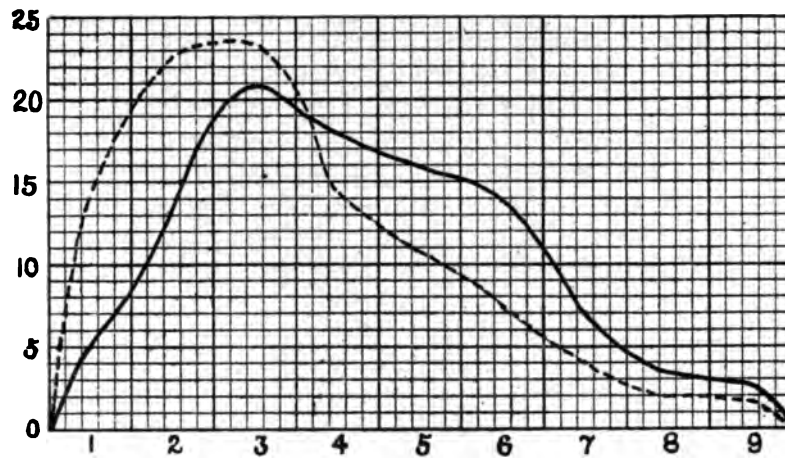


CHART 6. Combined curve of the five protein experiments.

The nitrogen curves obtained in these experiments do not differ greatly from those reported by others for normal dogs, though but few of the many protein feeding experiments previously published have been made under very similar experimental conditions. The work of Feder, Graffenberger, Stauber, Wolf, Mendel and Lewis, and others may be referred to. It is evident that the phlorhizinized kidneys are still capable of performing their normal function of excreting the end-products of nitrogen metabolism.

It seems remarkable that the glucose curve in most cases reaches its maximum *before* the nitrogen curve. An explanation for this may be in the fact that the nitrogenous portion of the protein is catabolized to its normal end products, while the non-nitrogenous has its metabolism arrested at the stage of glucose, an intermediary product. It is possible, however, that the greater permeability of the phlorhizinized kidney for glucose may be the true explanation of this phenomenon.

The digestion of proteins is seen to be, if we take the glucose curve as an index, still more rapid than the nitrogen curve would indicate. With figures which represent the mean of all five partition experiments here reported, 35 per cent of the extra glucose was found to be eliminated by the end of the second hour against 18 per cent of the nitrogen.

The glucose nitrogen ratio becomes very much increased in the first few hours, due not only to the greatly increased excretion of glucose, but also to the lack of coincidence of the glucose and nitrogen curves. The gliadin experiment well illustrates the fact that the size of the G:N ratio of the experimental period depends not only on the nitrogen and glucose formed from the ingested substance, but also on the time included in this period *before* and *after* the excretion of the extra nitrogen and glucose has begun and ended. This has been frequently overlooked in earlier work done on phlorhizin diabetes. It is illustrated by the following table from the gliadin partition. Time, as noted, begins with the ingestion of the protein. Mean G:N of the fore-and after-periods is 3.20. The calculated extra sugar remains, however, about the same for each period.



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PERIODS	GEN RATIO	EXTRA GLUCOSE
<i>hrs.</i>		
9	4.09	86.7
12	3.88	84.8
24	3.65	84.7

The present partitions seem to be the only ones on record where isolated proteins have been administered to fasting phlorhizinized dogs. Similarly planned experiments have, however, been carried out by Reilly, Nolan, and Lusk<sup>11</sup> in three experiments with feeding of meat. The results obtained are essentially the same as here reported, though the nitrogen and glucose of the meat were excreted somewhat more slowly. This is probably due to the relatively larger amounts fed.

A uniform plan has been adhered to in carrying out the above experiments. The curves of the nitrogen and glucose are similar in all cases, and represent the time taken for excretion of the products of both the nitrogenous and non-nitrogenous portions of the ingested protein. *This, it is believed, makes it clear that the rate of metabolism in the animal organism is, under optimal conditions, alike for both vegetable and animal proteins.*

Proteolytic enzyme action is mainly a matter of simple hydrolysis. It would seem improbable, *a priori*, that a difference in the behavior of pure proteins introduced into the body in a highly assimilable condition, whether of animal or vegetable origin, should evince itself. Vegetable proteins ingested in an impure state with cellulose, fiber, and other impurities are, indeed, as experience has shown, often less digestible than are animal proteins of softer texture. That this difference of digestibility does not depend upon chemical structure of the protein, the work here reported seems clearly to indicate. It must be emphasized, however, that the experiments here presented were made in the presence of the urgent need of the body for protein food and with excess of enzymes. It must be admitted, then, as possible that differences in the digestion rate of various proteins may occur under less favorable conditions.

<sup>11</sup> F. H. Reilly, F. W. Nolan, and G. Lusk: *Am. Jour. Physiol.*, i. p. 395, 1898.

## PART II. METABOLISM OF PROTEINS IN THE PHLORHIZINIZED ORGANISM AND RELATED QUESTIONS.

The amounts of extra glucose yielded by a series of eight proteins has been determined in the following experiments. They have been carried out under the same plan as that of those already described, except that twelve or twenty-four hour periods were employed. In view of the data already presented it seems certain that all the extra glucose and nitrogen were eliminated well within these periods of time.

The reliability of the results of these and other experiments in phlorhizin diabetes depends upon accepting the statement that the extra sugar originates from the substance ingested. Direct proof of this is lacking. It seems, therefore, not out of place to discuss this subject before describing the results of the experiments.

*Origin of the extra glucose in phlorhizin diabetes.*

For the rise in glucose elimination following ingestion of various substances by phlorhizinized animals, there can be but the following possible sources. The extra glucose has its origin either in the carbohydrates, fats, or proteins of the body, or in the ingested substance.

All previous experimental evidence demonstrates the rapid removal of carbohydrates at the beginning of the fasting phlorhizin period. The relation of the body glycogen to this question has been fully discussed by Lusk. It may suffice to state that although a small amount of glycogen and possibly of other carbohydrates persistently remains in the tissues of the fully phlorhizinized fasting animal,<sup>12</sup> no evidence has been advanced demonstrating that the ingestion of small portions of glucose-yielding substances can either lead to an increase in, or an elimination of, these carbohydrate rests. The breakdown of fat is without influence on the sugar excretion (Lusk and others). During the entire phlorhizin starvation period, breakdown of body protein leads to the elimina-

<sup>12</sup> 0.06 per cent liver (Pflüger and Junkersdorff). According to unpublished analyses made by Mr. Frank C. Csonka in this laboratory, the muscles of fasting phlorhizinized dogs contained 0.03 per cent glycogen and 0.56 per cent total substances reducing Fehling's solution.

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tion of the resulting glucose in definite ratio with a corresponding amount of nitrogen. It seems reasonable, therefore, to suppose that the corresponding amount of nitrogen would be eliminated should the extra sugar appearing after the ingestion of a given substance be derived from body protein. A long series of experiments is on record in which the ingested substance, which itself contained no nitrogen, led to extra sugar formation without any appreciable rise in nitrogen elimination. These experiments may be induced as indirect evidence against the origin of the extra glucose from body protein.

In support of the view that the extra glucose is formed from the ingested substance, the following may be mentioned. (1) Glucose fed to phlorhizinized dogs in the same amounts as the extra sugar excreted after feeding of glucogenetic substances, reappears quantitatively in the urine. It is thus made probable that glucose arising in the organism in like amount would also be eliminated quantitatively. (2) In no case, in the author's knowledge, roughness of calculation of the extra sugar being considered, has the extra sugar found been greater in amount than that derivable from the carbon of the compound ingested. (3) Administration of substances, incapable in view of their chemical structure of being converted into glucose, has been found not to be followed by extra glucose formation. (4) In the case of ingested proteins and other glucogenetic substances containing nitrogen, the extra glucose elimination rises and falls nearly parallel with an extra nitrogen elimination, as shown in partition experiments; from which it may be inferred that the origin of this extra glucose and nitrogen is in the ingested substance. (5) Evidence showing that the extra sugar derives its origin from other sources than the substance ingested has not been advanced; and, indeed, data tending to disprove that the origin of the extra sugar is in the ingested substance seems also to be lacking.

*Glucose yields of ingested proteins.*

PROTEIN	CASEIN	OVALBUMIN	SERUM ALBUMIN	GELATIN	FIBRIN	EDZESTIN	GLIADIN	ZEIN
Glucose yield in per cent .....	48	54	55	65	53	65	80	53

The percentages of protein converted by the organism into glucose represent the average of from four to eight experiments in each case. The results of the individual experiments did not show very wide variations. *The amounts of glucose derivable from the proteins examined are seen to represent from one-half to four-fifths of the weight of the ingested protein. From the standpoint of amount at least, dextrose must thus be considered one of the chief intermediary products of protein metabolism, and of certain proteins the most important.*

*Results of previous experiments.*

Of similar experiments already on record, but few may safely be used as a basis for calculation of extra sugar yields. The second of two gelatin experiments by Lusk<sup>13</sup> has been recalculated precisely according to the method employed in the experiments reported in this article. The yield obtained is 64 per cent. Halsey obtained yields of 49 per cent for casein and 50 per cent (recalculated) for fibrin. Lower glucose values in other of Halsey's casein experiments are probably due to the relatively large amounts of this material fed.

*Relation of the chemical constitution of proteins to their glucose yields in the phlorhizinized organism.*

In view of the studies of Embden, Lusk, Dakin, and others, the amino-acids of proteins may be classified as to their behavior in the glycosuric organism according to the following scheme.<sup>14</sup>

<i>Amino-acids.</i>	
GLUCOGENETIC	NON-GLUCOGENETIC
Glycine	Valine
Alanine	Leucine
Serine	Isoleucine
Cystine	Lysine
Aspartic acid	Phenylalanine
Glutaminic acid	Tyrosine
Ornithine	Tryptophane
Proline	

Of the non-glucogenetic amino-acids, leucine, tyrosine, and phenylalanine yield an increase of aceto-acetic acid (Baer and

<sup>13</sup> Reilly, Nolan, and Lusk: *loc. cit.*

<sup>14</sup> Dakin: *loc. cit.* See table.

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Blum, Ringer and Lusk, Dakin). The remainder of this group have not been clearly demonstrated to yield either glucose or aceto-acetic acid.

Ingested protein has been shown to break down rapidly into its component amino-acids; so it may be said that the metabolism of protein and amino-acids is, after this stage, practically the same. One might, therefore, expect to be able to calculate fairly definitely the amount of glucose a given protein should yield. Various reasons render this impossible. The analyses of hydrolyzed proteins are very incomplete, as only about 48 per cent, according to Osborne, of the amino-acids can be actually determined. Even if this great source of error could be eliminated, the sum of the amino-acids obtained from a given protein would not represent the exact amount of the protein analyzed; for they contain, in addition, water acquired by hydrolysis. Again, all proteins are not composed entirely of linked amino-acids. These acids have, moreover, been shown to yield amounts of glucose in the diabetic organism, which vary widely, from 93 per cent (alanine) to 62 per cent (aspartic acid) by weight.

An attempt, however, has been made to establish a basis of comparison. The results obtained with the method used are certainly of comparative value. The calculation is made as follows: As determined by the usual methods the amino-acids form, in general, a fairly representative fraction of the total amount of these acids present in the protein. There are found, of course, larger percentages of some than of others. As this is true for the glucogenetic as well as the non-glucogenetic acids, a certain, if very rough, balancing of this source of error evidently exists. The percentage relationship of the glucogenetic amino-acids with the total amino-acids as determined by analyses approximates that existing between the glucogenetic portion of the protein molecule and the entire molecule. The ratios so obtained have been graphically represented in the following figure, and for the sake of comparison the actual yields of glucose in per cent have been drawn to the same scale.

Though discrepancies occur, as would be expected from the rough mode of calculation, *the results as tabulated demonstrate in most cases a definite correspondence between the calculated glucose ratios and the values found by direct experiment. When the total*

*amounts of amino-acids as obtained by hydrolysis represent two-thirds or more of the protein, as is the case for casein, edestin, gliadin, and zein, this correspondence is very close, less than 5 per cent variation being observed.*

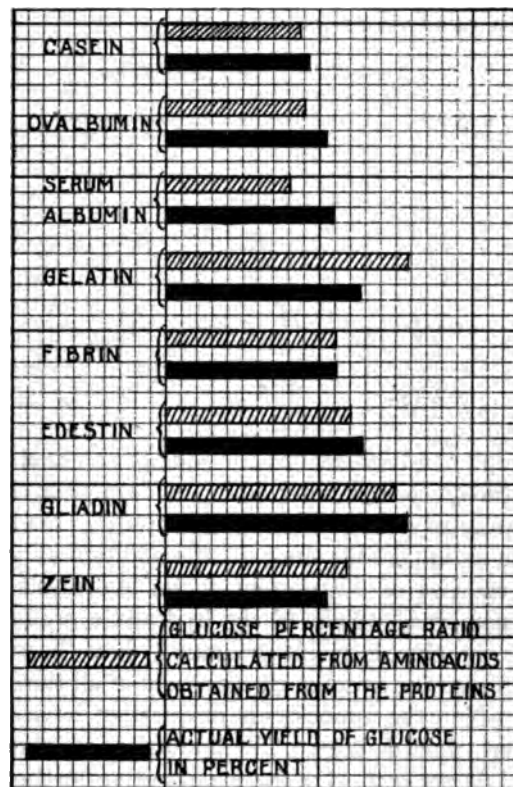


FIG. 1. Glucose derived from protein.

For the cases of widest variation from the calculated values, it is probable that the great incompleteness of the analyses is responsible. Thus, gelatin, which is peculiar in containing no tyrosine nor tryptophane, much proline, and glycine in greater abundance than almost any other protein, may be expected to yield considerable glucose. The high calculated ratio (see Figure 1) is, however, evidently due to the relative preponderance of gly-

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cine and proline in the *fraction* (45 per cent) of the amino-acids of gelatin accounted for by hydrolysis.

Gliadin forms more sugar in the organism than any protein examined. Fortunately, in this case, the amino-acids as determined by hydrolysis show a total of 84 per cent, which makes more definite calculations possible.

*Analysis of gliadin, 100 grams.*

GLUCOGENETIC AMINO- ACIDS PRESENT	ANALYSIS OF OSBORNE	PROBABLE ACTUAL AMOUNTS PRESENT	CALCULATED GLUCOSE YIELD
Alanine.....	2.0	4.3	4.0
Serine.....	0.2	3.0	2.6
Cystine.....	0.5	1.0	0.7
Aspartic acid.....	0.6	1.4	0.9
Glutaminic acid.....	43.7	63.3	42.1
Proline.....	13.2	21.7	15.8
Arginine.....	3.2	3.5	2.0
Total.....	63.4	98.2	68.1

The attempt has been made to calculate the probable actual amounts of the glucose-yielding amino-acids present in gliadin.<sup>15</sup> They total to nearly 100 per cent (column 2). This is, however, by no means an impossible sum; for the addition of water to the amino-acid radicals of the protein on its hydrolysis greatly increases the combined weight of the hydrolytic products. From these values the glucose yields (column 3) have been computed, using in each case the average glucose yield of all the experiments reported in which the amino-acids in question have been fed to fasting phlorhizinized dogs. The amounts of glucose so calculated total *68 per cent*. By the comparative method described above the calculated glucose is *76 per cent*. The actual average sugar yield obtained from gliadin by direct experimentation is *80 per cent*. Taking into account the rough mode of calculation necessarily employed, the results in all cases show no greater deviation from each other than might be expected. The yield of glucose from protein undergoing normal digestion might

<sup>15</sup> For the data employed see T. B. Osborne and D. B. Jones: *Am. Jour. Physiol.*, xxvi, p. 305, 1910. D. D. Van Slyke: this *Journal*, ix, pp. 185 and 205, 1911; x, p. 15, 1911-12. Cathcart: *loc. cit.*

fairly be expected to be relatively higher than that from isolated amino-acids, some of which are distinctly toxic when introduced into the organism in any considerable amount. For further calculations the following data have been employed:

According to Osborne,

100 gm. gliadin.....C=52.7 gm. H=6.9 gm. O=21.7 gm.

N=17.7 gm. S=1.0 per cent.

80 gm. glucose.....C=32.0 gm. H=5.4 gm. O=42.6 gm.

It has been calculated that 61 per cent of the carbon of gliadin leaves the body in the form of glucose. Judging from the oxygen contained in 100 grams of gliadin, 22 grams, and that contained in 80 grams of glucose, 43 grams, it would seem impossible that 80 grams of glucose could be formed from 100 grams of gliadin. This additional oxygen may, however, be accounted for by the process of hydrolysis, and oxygen may further be added in the process of synthesis of glucose from the products of protein digestion. 100 grams of the elemental components of gliadin may be approximately accounted for as follows: 59 grams of carbon, hydrogen, and oxygen combined in glucose, 18 grams as urinary nitrogen. The remaining 23 grams as yet unaccounted for consist chiefly of leucine, tyrosine, and phenylalanine, which yield no glucose. But aceto-acetic acid in the diabetic organism totals 12 per cent of the entire amount of amino-acids obtained from gliadin. It seems probable, then, that some of the residual carbon may go to form aceto-acetic acid and related bodies.

*The ratio glucose: nitrogen of proteins.*

PROTEIN	CASEIN	OVALBUMIN	SERUM ALBUMIN	GELATIN	FIBRIN	EDESTIN	GLIADIN	REIN
G: N ratio .....	3.08	3.49	3.48	3.59	3.16	3.48	4.53	3.29

The above table has been calculated from the percentage of nitrogen in the proteins and the glucose yielded by them in the phlorhizinized organism. With the single exception of gliadin, it is seen that the G:N ratios thus calculated vary to no greater extent than do the urinary G:N ratios of any extended series of fasting phlorhizinized dog experiments. The average ratio



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for fibrin, gelatin, and serum albumin, all proteins of wide distribution in the adult animal, is 3.41,—considerably lower than the G:N ratio for body protein, 3.67, established by Lusk. No certain conclusion can, however, be drawn from results representing but three proteins of the great number present in the animal body.

#### *Protein minimum and growth considerations.*

The interesting work of Michaud<sup>16</sup> and Zisterer<sup>17</sup> has shown that the least quantity of protein (the protein minimum) which must be digested in order to prevent loss of body protein varies according to the nature of the protein fed. The protein minimum is least for those proteins nearest in composition to "*Körpereigenes Eiweiss*." In order to make a comparison possible between the size of Zisterer's protein minima for the dog and the glucose yielded by ingested proteins in the same animal, when phlorhizinized, these values for casein have been taken as units, and the following graphic representation has been made.

The glucose yields are seen to correspond roughly to the size of the protein minima. The latter in the case of casein is less than that of the other proteins. The glucose produced in the organism by this protein approximates that of dog, fish, and ox muscle.<sup>18</sup> From these data the inference may be drawn that edestin and, in still greater degree, gliadin contain a balance of the various amino-acids less suitable for utilization in the dog than that found in casein, and that their glucose yields in the diabetic animal may be taken as an index of this unfavorable constitution.

But the problem of the nutritive value of food proteins is more complex than the above would indicate. The recent important work of Osborne and Mendel<sup>19</sup> has demonstrated in the most convincing manner that both maintenance of body weight and growth in animal depend directly on specific amino-acids, tryp-

<sup>16</sup> L. Michaud: *Ztschr. f. physiol. Chem.*, lix, p. 405, 1909.

<sup>17</sup> J. Zisterer: *Ztschr. f. Biol.*, liii, p. 157, 1909.

<sup>18</sup> Unreported experiments.

<sup>19</sup> T. B. Osborne, L. B. Mendel, and E. L. Ferry: *Ztschr. f. physiol. Chem.*, lxxx, p. 307, 1912; this *Journal*, xvii, p. 325, 1914 (where reference to other papers is made).

tophane being necessary for maintenance of body weight and lysine for growth. Neither of these amino-acids yield glucose. The glucose yields of the proteins in the diabetic organism could not, then, be expected to bear any close relationship to growth problems. Casein, ovalbumin, and zein yield amounts of extra glucose which do not vary greatly from one another. Casein and ovalbumin are rich in lysine and promote maximal growth, while zein fails to do so on account of its lack of lysine and tryptophane. The conclusion may be drawn from these and other

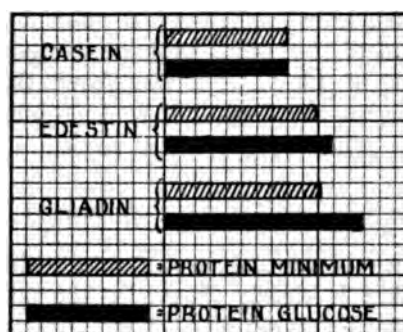


FIG. 2.

examples that the amounts of glucose yielded by the metabolism of proteins stand in no obvious relationship to their ability to promote growth.

*The sparing of body protein effected by the ingested proteins.*

A detailed study of this subject is beyond the scope of the present article. The method of calculation of the body protein spared by the ingested proteins was the usual one. From the average nitrogen excretion for the immediately preceding and succeeding periods, the amount of nitrogen eliminated in the experimental period in excess of that contained in the ingested substance was deducted. The sum so obtained, expressed in per cent of the average nitrogen excretion, is accepted as the per cent of body protein spared by the fed protein. Wide variations in the amount of body protein spared in different experiments

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were observed. The following table records the average results for each series of experiments.

CASEIN	OVALBU- MIN	SERUM ALBUMIN	GELATIN	FIBRIN	WHEATIN	GLIADIN	ZEIN
41	39	33	22	27	16.5	26	33

Casein and ovalbumin spare somewhat more protein than the other proteins, possibly because their composition may be more adapted to the general requirements of the body for amino-acids (Figure II). No marked difference is noted in the ability of the vegetable and of the animal proteins to spare body protein. In an earlier part of this paper it was shown that no difference was demonstrable in the digestibility of proteins of animal or vegetable origin. These facts stand in accord.

#### *Relationship of protein to carbohydrate metabolism.*

The importance of glucose as an intermediary product of protein metabolism is clearly shown by the data presented in this article. Fifty to 80 per cent of the ingested proteins and about 59 per cent of body proteins (Lusk) are converted into glucose in the phlorhizinized organism. A majority of the amino-acids occurring in proteins goes over into glucose. This formation of large amounts of glucose in protein metabolism may be considered a normal process.<sup>20</sup>

The important protein-sparing function of carbohydrate is known. It is also a well demonstrated fact that utilization of protein in some manner practically depends upon the presence of carbohydrate. The experimental work dealing with this subject has been collected and ably presented by Cathcart.<sup>21</sup> Dakin has shown that oxyaldehydes are probably very important metabolic substances related closely to glucose and to amino-acids as well. It could well be that glucose and amino-acids are broken down to oxyaldehydes, which add ammonia and form new amino-acids. Embden and Schmitz were able to demon-

<sup>20</sup> See Lusk: *loc. cit.*

<sup>21</sup> Cathcart: *loc. cit.*, p. 116, ff., (see p. 1, note 1).

strate the synthesis of an amino-acid (alanine) in the perfused glycogen-rich liver, on the addition of ammonium chloride to the perfused fluid. *Other indirect evidence could be mentioned, making it probable that protein may be synthesized from metabolic products closely related to glucose on the one hand, and simple nitrogenous compounds on the other.*

With the help of this view the apparent urgent need of the starving organism for carbohydrate may be explained. Landergren found that when the starvation excretion of nitrogen had been established, this minimum could be still further depressed by an exclusively carbohydrate diet, whereas a diet composed of fats employed under the same conditions did not have this effect. It is evident from this work that the organism in order to obtain carbohydrate even sacrifices an increased amount of its body protein. Aside from dynamic questions, this fundamental requirement of the starving body for glucose may be but an expression of its need for protein repair. Certain more vital organs are better sustained in starvation than other less important ones. It may be that the proteins of the latter are broken down successively to amino-acids, glucose, oxyaldehydes, and ammonia, from which the repair requirements of the vital proteins can be satisfied by synthesis of the special amino-acids needed. The introduction of ingested glucose limits protein breakdown, as it represents material suitable to protein synthesis. Osborne and Mendel<sup>22</sup> have successfully maintained animals, which have even raised young, on a diet containing a single isolated protein. The large number of protein syntheses which must here take place can certainly be easily explained by the above view of protein metabolism.

#### SUMMARY.

*Part I.* Vegetable and animal proteins under optimal conditions are metabolized at the same rate in the animal organism. All the extra glucose and nitrogen are eliminated by the ninth hour after ingestion.

*Part II.* See italics, first page of this article.

<sup>22</sup> Osborne and Mendel: this *Journal*, xiii, p. 233, 1912-13.

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## PROTOCOLS.

The protocols of the longer periods of these experiments are grouped with the succeeding ones. In this series the proteins were fed within the first six minutes of the first hour of the partition period.

PERIODS	NITROGEN	GLUCOSE	NITROGEN	GLUCOSE	G:N	
hrs.	gm.	gm.	HOURLY	HOURLY		
8-9 a.m.....			0.408	3.390	8.31	Casein Experiment 1. At 8 a.m. 25.54 gm. (N=3.88 gm.) casein with 350 cc. water.
9-10 a.m.....			0.505	3.950	7.83	
10-11 a.m.....			0.624	3.623	5.80	
11 a.m.-12 m..			0.467	2.691	5.76	
12 m.-1 p.m..			0.442	2.353	5.33	
1-2 p.m.....			0.449	2.146	4.78	
2-3 p.m.....			0.378	1.899	5.02	
3-4 p.m.....			0.340	1.508	4.72	
4-5 p.m.....			0.336	1.543	4.59	
5-8 p.m.....	0.981	3.984	0.327	1.328	4.06	
8-11 p.m.....	0.981	4.000	0.327	1.333	4.07	Casein Experiment 2. At 8 a.m. 16.77 gm. casein (N=2.56 gm.) with 230 cc. water.
11 p.m.-2 a.m.	1.128	3.922	0.376	1.307	3.48	
2-8 a.m.....	2.229	7.519	0.371	1.253	3.37	
8-9 a.m.....			0.282	1.117	4.16	
9-10 a.m.....			0.408	1.887	4.63	
10-11 a.m.....			0.496	2.091	4.21	
11 a.m.-12 m..			0.467	1.754	3.76	
12 m.-1 p.m..			0.408	1.415	3.47	
1-2 p.m.....			0.437	1.167	2.73	
2-3 p.m.....			0.303	0.935	3.09	
3-4 p.m.....			0.265	0.791	2.98	At 9 a.m. 29.29 gm. serum albumin (N=4.60 gm.) moistened with a little water.
4-5 p.m.....			0.322	0.805	2.50	
5-8 p.m.....	0.925	2.049	0.308	0.683	2.22	
8-11 p.m.....	0.743	2.475	0.248	0.825	3.33	
11 p.m.-12 m.	0.729	2.000	0.243	0.666	2.74	
2-8 a.m.....	1.279	4.167	0.213	0.695	3.26	
9-10 a.m.....			0.465	2.113	4.54	
10-11 a.m.....			0.528	2.508	4.56	
11 a.m.-12 m.			0.534	2.351	4.32	
12 m.-1 p.m..			0.646	2.164	3.30	
1-2 p.m.....			0.729	2.222	3.00	
2-3 p.m.....			0.708	2.066	2.92	
3-4 p.m.....			0.580	1.655	2.85	
4-5 p.m.....			0.530	1.500	2.83	
5-6 p.m.....			0.450	1.364	3.03	
6-9 p.m.....	1.072	3.540	0.357	1.180	3.30	
9 p.m.-12 m..	1.080	3.393	0.360	1.131	3.14	
12 m.-3 a.m..	1.088	3.695	0.362	1.232	3.40	
3-8 a.m.....	1.745	5.334	0.349	1.066	3.06	

## PROTOCOLS—Concluded.

PERIODS	NITROGEN	GLUCOSE	NITROGEN HOURLY	GLUCOSE HOURLY	G:N	
hrs.	gm.	gm.	gm.	gm.		
8-9 a.m.....			0.488	2.826	5.79	At 8 a.m. 17.48 gm. gliadin (N=3.07) in 150 cc. $\frac{N}{10}$ NaOH followed by equal volume of $\frac{N}{10}$ HCl.
9-10 a.m.....			0.513	3.343	6.52	
10-11 a.m.....			0.746	4.271	5.73	
11 a.m.-12 m.			0.828	3.127	3.77	
12 m.-1 p.m....			0.858	3.032	3.53	
1-2 p.m.....			0.718	2.371	3.30	
2-3 p.m.....			0.660	1.973	2.99	
3-4 p.m.....			0.575	1.734	3.02	
4-5 p.m.....			0.498	1.750	3.52	
5-8 p.m.....	1.521	4.645	0.507	1.548	3.05	
8-11 p.m.....	1.395	4.279	0.465	1.426	3.06	
11 p.m.-2 a.m.	1.598	5.000	0.533	1.666	3.13	
2-5 a.m.....	1.416	4.828	0.472	1.609	3.41	
5-8 a.m.....	1.591	4.990	0.530	1.663	3.14	
7-8 a.m.....			0.412	1.225	2.97	At 8 a.m. 16.90 gm. edestin (N=3.18 gm.) with 270 cc. H <sub>2</sub> O.
8-9 a.m.....			0.508	2.230	4.43	
9-10 a.m.....			0.811	3.509	4.33	
10-11 a.m.....			0.863	3.175	3.68	
11 a.m.-12 m.			0.653	1.887	2.89	
12 m.-1 p.m..			0.488	1.250	2.56	
1-2 p.m.....			0.453	1.124	2.48	
2-3 p.m.....			0.410	1.079	2.63	
3-4 p.m.....			0.391	1.183	3.02	
4-5 p.m.....			0.371	1.005	2.71	
5-8 p.m.....	1.194	2.871	0.398	0.957	2.40	

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QUANTITY FED	NITROGEN IN SUBSTANCE FED	WEIGHT OF DOG	PERIOD	NITROGEN	GLUCOSE	G:N	EXTRA GLUCOSE	
gm.	gm.	kgm.	hrs.	gm.	gm.		gm.	per cent
Casein, 56.8...	{ 8.88 (1.0 gm. per kgm.) }	8.8	12	4.30	15.05	3.50		
			24	13.08	45.61	3.43	31.2	54.9
			12	4.30	13.23	3.07		
			12	3.82	12.93	3.38		
Casein, 25.0...	3.90	6.6	24	6.87	19.62	2.86		
			24	9.88	27.89	2.82	10.4	41.6
			24	8.18	22.44	2.74		
			24	6.64	18.17	2.85		
Casein, 28.4...	{ 4.44 + 0.06 gm. beef ex- tract N = 4.50 }	7.5	12	3.18	11.05	3.38		
			24	7.86	27.48	3.50	15.89	55.9
			24	5.61	18.41	3.28		
Casein, 54.2...	{ 8.47 + 0.14 beef extract N = 8.61 }	14.3	12	11.44	35.21	3.15		
			12	10.31	31.27	3.03		
			24	24.85	73.14	2.94	23.12	42.6
			24	18.57	58.08	3.12		
Casein, 23.1...	{ 3.61 + 0.09 beef extract N = 3.70 }	5.7	12	3.91	11.87	3.04		
			24	8.02	24.23	3.02	12.13	52.5
			24	6.05	16.08	2.66		
Casein, 34.7...	5.28	9.2	24	9.12	29.74	3.26		
			24	9.36	31.85	3.33		
			24	11.08	35.80	3.22	16.49	47.5
			24	7.87	26.19	3.33		
Casein,* 25.54...	3.88	9.7	12	3.92	15.33	3.91		
			12	3.91	15.73	4.02		
			12	4.93	27.09	5.49	11.53	45.1
			12	4.34	15.44	3.29		
Casein,** 16.77...	2.56	6.4	12	5.32	16.28	3.05		
			12	2.86	8.66	3.06		
			12	3.05	8.20	2.74		
			12	4.31	12.64	2.93	7.33	43.7
			12	2.75	8.64	3.14		

\* Casein Partition Experiment 1. In this experiment the nitrogen excretion is so irregular that the extra glucose was calculated from the sugar values alone, which are unusually regular.

\*\* Casein Partition Experiment 2.

SUBSTANCE FED	NITROGEN IN SUBSTANCE FED	WEIGHT OF DOG	PERIODS	NITROGEN	GLUCOSE	G:N	EXTRA GLUCOSE
gm.	gm.	kgm.	hrs.	gm.	gm.		gm. per cent
Ovalbumin,* 31.70...	4.52	8.6	12	3.85	15.65	4.06	17.40 54.9
			12	3.92	15.40	3.93	
			24	8.80	34.48	3.92	
Ovalbumin, 27.66...	4.30	8.6	12	3.55	12.79	3.60	14.50 52.14
			12	4.05	13.61	3.37	
			24	9.44	31.79	3.37	
Ovalbumin, 18.00...	2.80	5.6	24	8.00	26.49	3.31	10.51 58.4
			12	2.59	7.71	2.97	
			12	2.54	7.51	2.96	
Ovalbumin, 31.42...	4.80	9.6	24	5.80	18.85	3.25	15.95 50.8
			12	4.37	11.36	2.60	
			12	5.42	19.08	3.52	
Serum albumin, 34.12	5.20	10.4	12	4.71	17.19	3.65	18.80 55.1
			12	10.26	34.55	3.37	
			12	4.18	13.90	3.33	
Serum albumin, 30.76	4.85	9.7	12	3.76	11.88	3.16	15.30 49.7
			24	6.94	23.26	3.35	
			24	13.68	46.87	3.43	
Serum albumin, 21.93	3.45	6.9	24	10.98	36.10	3.29	12.87 58.7
			24	13.19	41.24	3.13	
			24	9.29	27.21	2.93	
Serum albumin, 27.37	4.28	8.8	24	8.02	27.08	3.39	15.25 55.7
			24	9.31	32.79	3.52	
			24	7.34	24.91	3.40	
Serum albumin,** 29.29...	4.60	9.2	24	7.90	24.97	3.16	15.90 53.7
			12	9.57	31.75	3.32	
			12	7.62	23.53	3.09	
Serum albumin,** 29.29...	4.60	9.2	12	4.73	17.01	3.60	15.90 53.7
			12	6.24	21.48	3.44	
			12	4.17	13.31	3.19	
Serum albumin,** 29.29...	4.60	9.2	12	4.04	13.89	3.43	15.90 53.7
			12	6.24	21.48	3.44	
			12	4.17	13.31	3.19	

\* In this experiment a partially purified preparation of ovalbumin (N=12.39 per cent) was employed. The other experiments were carried out with preparations as described in the text.

\*\* Partition experiment.



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SUBSTANCE FED	NITROGEN IN SUBSTANCE FED	WEIGHT OF DOG	PERIOD	NITROGEN		G:N	EXTRA GLUCOSE	
				gm.	gm.		gm.	percent
			24	9.29	27.21	2.93		
Gelatin, 25.87.....	4.55	9.1	24	11.26	36.70	3.25	17.78	68.7
			24	7.10	19.42	2.72		
			24	13.14	44.45	3.38		
			24	13.77	45.98	3.34		
Gelatin, 38.41.....	6.80	13.6	24	15.90	56.43	3.54	24.40	63.5
Gelatin, 22.37.....	3.96	13.2	24	12.45	43.32	3.48	13.44	60.1
			24	9.26	35.81	3.87		
			24	12.19	41.24	3.38		
Gelatin, 27.93.....	4.95	9.9	24	13.22	47.34	3.58	18.85	67.5
			24	9.72	34.07	3.51		
			12	5.36	17.75	3.31		
Gelatin, 12.80.....	2.25	4.5	12	6.23	20.30	3.26	8.12	63.4
			12	4.55	12.80	2.82		
			24	7.08	20.36	2.88		
			24	6.28	18.26	2.91		
Fibrin, 21.40.....	3.55	7.1	24	7.79	24.69	3.17	12.01	56.1
			24	5.80	17.83	3.07		
Fibrin, 19.89.....	3.30	6.6	24	7.16	23.46	3.28	10.00	50.3
			24	4.95	19.37	3.91		
			24	10.82	35.09	3.24		
			24	10.43	36.47	3.50		
Fibrin, 30.45.....	5.05	10.1	24	12.84	42.85	3.34	16.59	54.5
			24	5.72	21.56	3.76		
Fibrin, 15.06.....	2.5	5.0	24	6.76	22.99	3.40	7.91	52.5
			24	4.81	16.04	3.33		
			24	6.43	25.56	3.97		
			12	2.98	12.65	4.25		
Edestin, 16.00.....	3.00	6.0	24	7.42	27.70	3.73	9.54	59.6
			24	3.98	16.67	4.18		
Edestin, 15.47.....	2.90	5.8	24	6.59	26.22	3.96	10.61	68.6
			24	4.31	18.45	4.28		
			24	4.31	16.52	3.83		
			24	10.19	36.53	3.58		
Edestin, 26.40.....	4.95	9.9	24	11.57	41.33	3.57	17.30	65.5
			24	8.60	34.62	4.02		
			24	8.03	29.23	3.64		

SUBSTANCE FED	NITROGEN IN SUBSTANCE FED	WEIGHT OF DOG	PERIOD	NITROGEN	GLUCOSE	G:N	EXTRA GLUCOSE	
gm.	gm.	kgm.	hrs.	gm.	gm.		gm.	per cent
Edestin, 14.56.....	2.73	5.5	24	6.94	24.94	3.60		
			24	8.13	29.52	3.63	9.65	66.3
			24	5.72	21.56	3.76		
Edestin, 16.90.....	3.18	7.95	12	3.89	11.56	2.97		
			12	6.55	20.54	3.14	10.91	64.6
			12	4.15	11.46	2.76		
Gliadin,* 19.66.....	3.36	11.2	12	3.68	13.18	3.58		
			24	7.11	27.08	3.81		
			24	7.90	32.00	4.05	15.75	80.1
Gliadin,* 16.45.....	2.94	9.8	24	6.63	22.22	3.35		
			24	9.73	30.81	3.17		
			24	10.20	36.57	3.59	13.48	82.0
Gliadin, 22.6 .....	4.10	8.2	24	7.76	24.75	3.19		
			12	4.20	13.96	3.32		
			12	4.18	14.69	3.52		
Gliadin, 11.70.....	2.07	6.9	24	10.28	38.04	3.70	17.77	78.6
			24	8.48	26.69	3.15		
			12	4.61	11.74	2.55		
Gliadin,** 17.48.....	3.07	8.3	12	5.45	17.32	3.17	8.84	75.6
			12	4.05	9.99	2.47		
			12	6.84	22.36	3.27		
Zein,*** 20.68.....	3.36	10.3	12	7.49	29.07	3.88	14.83	84.8
			12	6.00	19.10	3.18		
			12	6.21	19.50	3.14		
Zein,*** 20.68.....	3.36	10.3	12	5.12	15.70	3.45		
			12	4.65	15.54	3.35		
			24	9.27	31.09	3.35	11.47	55.5
			24	8.75	28.44	3.25		

\* In these experiments gliadin was fed as moist boli on the back of the tongue. In the others it was dissolved in acid or alkali (see text). From 30 to 66 cc. of total fluid per kgm. were employed.

\*\* Partition experiment.

\*\*\* In this experiment the granulated zein was fed as moist boli on the back of the tongue. In the other experiments it was freshly precipitated, as described in the text.

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SUBSTANCE FED	NITROGEN IN SUBSTANCE FED	WEIGHT OF DOG	PERIODS	NITROGEN	GLUCOSE	G: N	EXTRA GLUCOSE
<i>gm.</i>	<i>gm.</i>	<i>kym.</i>	<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>		<i>gm. per cent</i>
Zein, 17.23 .....	2.80	5.2	12	3.98	12.58	3.16	
			12	3.81	11.43	3.00	
			24	7.02	21.28	3.03	8.64 50.2
			12	3.03	9.05	2.95	
			12	3.00	8.10	2.70	
Zein, 14.68 .....	2.39	4.9	12	3.15	9.05	2.87	
			12	2.89	7.57	2.67	
			24	5.84	16.95	2.90	7.26 49.4
			12	1.95	5.66	2.90	
			24	4.23	12.18	3.03	4.39 55.3
Zein, 16.55 .....	2.70	9.2	12	1.88	4.59	2.44	
			12	1.75	4.33	2.47	
			12	5.81	17.54	3.02	
			12	5.47	16.19	2.96	
			24	11.44	34.68	3.03	8.90 53.8
Zein, 15.08 .....	2.46	8.4	12	5.48	16.27	2.97	
			12	4.61	13.16	2.85	
			12	5.33	16.13	3.03	8.15 54.0
			12	4.37	11.56	2.64	
			12	4.16	11.05	2.65	
			12	3.80	10.65	2.92	

THE COMPARATIVE NUTRITIVE VALUE OF CERTAIN  
PROTEINS IN GROWTH, AND THE PROBLEM  
OF THE PROTEIN MINIMUM.<sup>1</sup>

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In earlier communications<sup>2</sup> we have pointed out the dominant importance of certain amino-acids in the problems relating to the function of the nitrogenous food intake in both maintenance and growth. When the content of any essential amino-acid group in a specific protein is relatively small, the comparative poverty in the amino-acid in question will not manifest itself so long as the diet contains a surplus of this amino-acid above either the maintenance or growth quota. If, on the contrary, the intake of the protein is kept *low*, a plane will ultimately be reached where the yield of the amino-acid in question becomes so small that it cannot satisfy, first, the growth requirement and, later, the maintenance need for the nutrient unit in question, even though the other amino-acids are still available in suitable quantity. With a constant energy intake the amount of protein available for constructive functions will be limited by the "law of minimum." For example, a diet containing 20 per cent of the calories ingested in the form of some protein relatively deficient in an essential amino-acid may supply enough of that amino-acid to satisfy the requirements of the animal for maintenance and growth; whereas

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> T. B. Osborne and L. B. Mendel: this *Journal*, xvii, p. 325, 1914; xviii, p. 1, 1914.

one containing only 10 per cent of its calories in the form of the same protein, with an equivalent energy intake, may not supply enough. To determine whether or not such deficiencies will be shown by experiments in which rats are supplied *ad libitum* with foods containing different percentages of protein, but of approximately the same calorific value, we have tried experiments the results of which are shown in the following charts in the appendix: I, with 18, 15, 12, 9, 6, 4.5, and 2 per cent of *casein*; II, with 18, 15, 12, 9, 4.5, and 2 per cent of *edestin*; and III, with 18, 11, 9, 4.5, 2.5, 2, and 1 per cent of *lactalbumin*.

To learn the cause of the slower growth on the diets containing the lower percentages of protein we have tried the effect of additions of *cystine* to the casein foods and of *lysine* to the edestin foods, with the results shown in Charts IV and V. On comparison with many other proteins casein doubtless yields relatively little cystine; for on treating with caustic alkali according to the method of Schulz<sup>3</sup> only one-eighth of the total sulphur is converted into sulphide. This proportion is much less than that yielded by most other proteins.<sup>4</sup> Thus, of the proteins used in our experiments, gliadin and conglutin yield about two-thirds, glycinin and edestin about one-half, zein, ovovitellin, and ovalbumin about one-third of their total sulphur as sulphide. Since, under the conditions existing in the analytical processes to which the proteins were subjected in making these determinations, cystine yields about two-thirds of its sulphur as sulphide, it is not improbable that from these figures the relative proportion of the cystine complex in these proteins can be calculated. Assuming that the sulphur which separates as sulphide corresponds to two-thirds of the sulphur of the cystine complex which they contain, casein should yield about 0.60 per cent of cystine, whereas ovalbumin should yield 3.0 per cent and gliadin 3.7 per cent. Attempts to isolate cystine directly from the proteins have thus far yielded only a small part of the total cystine which they probably should.

In order to determine the effect of adding an amino-acid to a food containing a protein yielding this amino-acid in a relatively small amount, it is necessary to know the least quantity of this

<sup>3</sup> N. Schulz: *Ztschr. f. physiol. Chem.*, xxv, p. 16, 1898.

<sup>4</sup> T. B. Osborne: *Jour. Am. Chem. Soc.*, xxiv, p. 140, 1902.

protein which will promote normal growth. Obviously if more is supplied the additions will have no effect on the rate of growth. Furthermore, definite conclusions can only be reached when the comparisons are made during periods in which the amount of food eaten is the same. In the experiments here described these conditions have only been approximately fulfilled, and it will be necessary to adopt a different procedure before satisfactory evidence can be obtained which will demonstrate in detail that which in general these experiments unquestionably show.

In regard to the minimum amount of casein which the food must contain in order to promote normal growth, we have established by numerous experiments that when the diet contains 18 per cent of casein, along with the essential non-protein components of the ration, young rats can complete their growth satisfactorily. We have brought such animals to full size and kept them in health on such a diet for more than 620 days. With 15 per cent of casein in the food growth was still made at a normal rate (see Rat 2465, Chart I), although the summary below shows that a smaller amount of casein was eaten:

RAT	FOOD	GREW		GAIN		ATE	
		from	to			Food	Casein
		gm.	gm.	gm.	dys.	gm.	gm.
1592	18% casein	83	287	204	119	1228	199
2465	15% "	77	286	209	119	1187	160

When the casein content is reduced to 12 per cent growth falls a little below the normal, as shown by Rat 2117, Chart IV. The failure of Rat 2117 to grow as well on the 12 per cent casein food as did Rat 2465 on the 15 per cent casein food might have been due to a too low food intake; or conversely the lower food intake might be attributable to the failure to grow and consequent need for less protein. That the former is not true is shown by the normal growth of Rat 2124, Chart IV, which had the same food as Rat 2117, but with an addition of cystine equal to 3 per cent of the casein.

RAT	FOOD	GREW		GAIN		ATE	
		from	to			Food	Casein
		gm.	gm.	gm.	dys.	gm.	gm.
2465	15% casein	103	208	105	56	498	67.0
2117	12% "	98	142	44	56	277	40.6
2124	12% " + cystine	101	181	80	56	388	41.1

Since the addition of cystine in this case rendered the 12 per cent casein food more efficient for growth, it is probable that the minimum proportion of casein in food mixtures of this character for normal growth lies somewhat below 15 per cent. In how far differences in the ability of individual rats to utilize their food for growth may modify such a conclusion can only be determined by further experiments.

When the casein is reduced to 9 per cent, growth is promptly limited by the protein factor. Our present studies<sup>6</sup> have made it obvious that this failure to grow at a rate equivalent to the normal is not attributable to too little protein in the diet. *The addition of isolated cystine to the food containing 9 per cent of casein, without any other supplement, at once renders the ration decidedly more adequate for growth.* This is well shown by comparison of Rats 2043, 2051, 2481, 2483, 2484 in Chart IV. Growth can be facilitated or repressed at will by the addition or withdrawal of the extra cystine from the diet containing 9 per cent of casein.

Obviously a progressively lowered intake of casein with its detrimental consequences for growth cannot be remedied indefinitely by supplementing it with cystine. A level is finally reached at which the possibility of further growth, or even of maintenance, is limited by the lack of many, or all, of the numerous necessary nitrogenous units. The protein intake as a whole

<sup>6</sup> The data recorded here are not directly comparable with those relating to limiting growth by diminishing the proportion of protein which were published earlier by us (*Ztschr. f. physiol. Chem.*, lxxx, p. 340, 1912), because the foods in the present series contained butter-fat, which has been shown to facilitate growth (this *Journal*, xvi, p. 423, 1913-14). In the former experiments lard was the sole fat used, and failure to grow invariably ensued sooner or later on these diets, quite independently of the content of protein present.

becomes inadequate. Other amino-acids are simultaneously needed. It will be noted in Chart IV that when the food contains only 6 per cent or 4.5 per cent of casein (Rats 2519, 2116), added cystine alone no longer suffices to facilitate growth as vigorously as it did in the case where 9 per cent of casein was present (Chart IV, Rats 2043, 2051, 2481, 2483, 2484).

Comparable phenomena were obtained when edestin formed the sole protein of the diet (see Chart V). Here, too, growth on a ration containing only 9 per cent of edestin has fallen behind that secured with larger proportions. That the limiting factor may be the comparatively low yield of lysine is indicated in the figures below:<sup>6</sup>

Lactalbumin, cow's milk.....	8.10 per cent
Casein, " " .....	7.61 per cent
Edestin, hemp-seed.....	1.65 per cent
Gliadin, wheat.....	0.16 per cent

As with casein, normal growth has been secured with foods containing 15 per cent of edestin. With 12 per cent the rate of growth was a little less than normal. That this proportion of edestin is somewhat too small is indicated by the slightly improved growth when lysine equal to 2 per cent of the edestin was added to the food. That this was not attributable to an increased food intake is seen from the following figures:

RAT	LYSINE	GREW		GAIN		ATE	
		from	to	gm.	dys.	Food	Edestin
		gm.	gm.			gm.	gm.
2176	0	53	129	76	70	435	48.5
2120	0	54	121	67	70	391	43.6
2119	+	51	144	93	70	389	43.4

From these data it would appear that the added lysine renders the edestin in the food containing 12 per cent slightly more available for growth, and that this proportion of edestin is somewhat too small to meet the full requirements of these animals, for none of them grew at the full normal rate (see Chart V).

<sup>6</sup> A more extensive tabular summary is given in our paper: this *Journal* xvii, p. 334, 1914.



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With 9 per cent edestin, as can be seen from Chart V, growth was less rapid than with 12 per cent. *The addition of lysine to the 9 per cent edestin food caused some improvement in growth.* This increased growth was relatively much greater than can be attributed to the increased food intake, especially when the greater requirement for food caused by increase in size of the growing rat is taken into consideration. Thus:

RAT	LYSINE	GREW		GAIN		ATE	
		from	to			Food	Edestin
		gm.	gm.	gm.	dys.	gm.	gm.
2110	0	59	84	25	42	239	20.0
2050	0	48	81	33	42	234	19.6
2110	0	93	126	33	42	264	22.1
2050	+	91	172	81	42	332	27.8

Comparing 2110 with 2050 in the second period, the latter made a gain in weight 145 per cent greater than that made by 2110, but ate only 22 per cent more food, despite its larger ultimate size.

The favorable results following the addition of lysine to the 9 per cent edestin food are not as striking as in the case of the cystine-casein experiment. It is, of course, not difficult to conjecture that the relative paucity of lysine groups in edestin is not as marked as is the comparatively great deficiency of the cystine in casein. In either case it is not more protein as a whole, *i.e.*, not *all* of the amino-acids that are required to make the constructive material adequate; though of course the deficiency can be supplied as well by raising the proportion of the protein itself as by additions of amino-acids.

*The relatively greater efficiency of lactalbumin in promoting growth is striking;* for with only 9 per cent in the food the rate of growth was about normal (Chart VI), and the lower percentages were in all cases far more efficient than the corresponding proportions of casein or edestin (Charts VII. and VIII). Owing to our present limited knowledge of the products of hydrolysis of lactalbumin no experiments were made with amino-acid additions. Such information as we have relating to its amino-

acid make-up shows that both lysine and tryptophane<sup>7</sup> are relatively abundant. The marked nutritive efficiency of lactalbumin is probably due to a more perfect balance in the proportions of the essential amino-acid groups which it contains.

*Relation of growth to food intake.* It may be objected fairly that some of the conclusions here drawn are rendered inconclusive by the uncertain factor of the total food intake of the rats. Obviously if an animal consumes twice as much of a 5 per cent protein food as of a comparable 10 per cent protein ration it will obtain precisely the same total amount of protein daily from these food mixtures of quite unlike percentage composition. To permit tenable conclusions it is necessary to ascertain the actual intake and calculate the absolute amounts of protein ingested. Rat 2051, Chart IV, for example, on a diet containing 9 per cent of casein gained 8 grams in body weight in a period of three weeks, with a food intake of 113 grams (9.2 grams of protein); on the diet containing 9 per cent of casein plus cystine the same rat gained 35 grams of body weight in a second period of three weeks on a food intake of 123 grams (10 grams of protein). In other words, the growth in the presence of cystine was nearly 400 per cent greater,—a gain which could hardly be accounted for by the 8 per cent increase in the amount of food consumed, and despite the need of more food on the part of the animal as it became larger in the second period. Again, Rat 2481 on food containing 9 per cent casein plus cystine, in 35 days ate 317 grams of food (containing 25.4 grams of protein) to grow from 97 to 164 grams, *i.e.*, to gain 67 grams in body weight; whereas Rat 2117 (Chart IV), on 12 per cent casein food in 84 days ate 584 grams (containing 63.2 grams of protein) to make essentially the same gain, *i.e.*, to grow from 98 to 162 grams. Expressed in still another way, during the five weeks in which Rat 2481 made the 67 grams of body increment on a cystine food intake of 317 grams, Rat 2117 gained only 38 grams on a food intake of 247 grams containing essentially the same amount of protein, *viz.*, 27 grams. A quantity of food of approximately the same order of magnitude

<sup>7</sup> Unpublished determinations of tryptophane from lactalbumin made in this laboratory have shown higher yields of this amino-acid than have been obtained from any other protein as yet thus examined.

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from the standpoint of its energy yield has produced better growth in various instances, summarized below:

RAT	INITIAL WEIGHT	WEIGHT GAINED	FOOD EATEN	DURATION OF OBSERVATION
	gm.	gm.	gm.	dys.
1655♂.....	91	58	237	35
1654♂.....	91	65	251	35
1615♂.....	93	50	253	35
1657♂.....	104	73	284	35
1592♂.....	98	80	285	35
1650♂.....	100	73	285	35

It is not always easy to dissociate the increased growth on a particular diet from the factor of increased food intake thereon; for when the animal actually increases in body weight it requires more food, and the comparison of the larger animal on the suitable diet with the smaller one on an unsuitable ration is almost impracticable. We have the impression that the effect of cystine is something more than a mere stimulus to appetite leading to greater food intake. This belief is fortified by the observation that the rats which grow well on 9 per cent casein plus cystine in the food frequently eat no more in terms of calories and ingest no more protein than do rats which are growing no better on even larger percentages of casein without the addition of cystine. It thus appears that a marked deficiency in any essential ingredient of the diet does not lead to a corresponding compensatory increase in food intake.<sup>8</sup>

The experience which we have gained in measuring the food eaten by many rats at all periods of their growth has given us the conviction that the intake of the individual is determined in large measure by the energy requirement at any given period.

<sup>8</sup> This fact may help to explain the nutritive failures recently reported by P. Tachau (*Biochem. Ztschr.*, lxxv, p. 253, 1914) for mice fed on rations in which a suitable diet was made unsuitable by extensive addition of non-protein components,—fats and carbohydrates. To us, the consequent lowering of the per cent of protein in the ration without a corresponding increment in food consumption to keep the protein up to the requisite minimum seems a likely explanation of Tachau's results. This is fortified by the fact that additions of protein to the unsuitable food produced improvement.

Aside from occasional extreme figures for food intake furnished by our records, this seems to be substantially true.

*Food intake and gains in body weight of rats growing on diets containing various percentages of casein.*

RAT	DIET	INITIAL BODY WEIGHT	GAIN			INTAKE OF	
						Total food	Protein
		gm.	gm.	dys.	gm.	gm.	
1655♂.....	18% casein	60	135	80	578	93.7	
1652♂.....	" "	60	141	80	667	108.1	
1592♂.....	" "	49	171	80	616	99.7	
2465♂.....	15% casein	75	151	80	697	94.1	
2466♂.....	" "	81	83	80	595	80.3	
2117♂.....	12% casein	45	95	80	510	55.1	
2481♂.....	9% casein + cystine	51	135	80	667	54.0	
2483♂.....	" " "	52	107	80	591	47.9	
2484♂.....	" " "	55	101	80	619	50.1	
2051♂.....	9% casein	42	59	80	435	35.2	
2519♂.....	6% casein + cystine	52	65	80	511	27.6	
2509♂.....	6% casein	53	31	80	440	23.8	
2506♂.....	" "	53	15	80	375	20.2	

These figures show the actual food intake of a series of growing rats at different stages of growth. They give some idea of the extent to which the preceding statement is valid. At any rate the animals do not consume *proportionately* more of the artificial ration because it happens to be decidedly poor in protein; but, roughly speaking, they apparently limit their feeding to the amount of food yielding approximately the requisite energy. They may even eat less of it than of the ration richer in protein. If we compare the intake of food containing 18 per cent or 15 per cent of protein with that of food containing only 9 per cent, but with addition of cystine, it is evident that in order to furnish the same quantities of protein in each case it would be necessary for the animal to eat respectively two or one and two-thirds times the amount of the low protein diet as of the rations higher in protein. This has not been the case, as the actual data above regarding food intake will show.

In harmony with the foregoing comments Hopkins has remarked: "Only those perhaps who have had the experience of feeding ani-

imals with excess of food, and have noted the amount eaten for considerable periods, will realize how well adjusted, under normal circumstances, is the instinctive appetite to the physiological needs."<sup>9</sup> Rubner<sup>10</sup> states that many years of experience with dogs leads him to believe that appetite and capacity for digestion and absorption depend on the dog's requirement for energy in his given state of nutrition. A diet which a dog will greedily devour when in a room at a temperature of 0°, he will in part refuse when at a temperature of 33°.

A study of the intake of food containing only 2 per cent of protein by the rats whose weight curves are plotted in Chart VIII shows that in terms of energy it is not widely different, for animals of a given size, from the intake of similar food, rich in protein, by animals of the same size which are growing satisfactorily. A few data may suffice for illustration:

*Food intake of rats on foods of varying protein concentration.<sup>11</sup>*

(Grams eaten per week.)

RAT	BODY WEIGHT	NATURE OF FOOD	FOOD INTAKE
	gm.		gm.
<i>Rats growing.</i>			
1650♂	85	18% edestin + protein-free milk + butter-fat	41
1652♂	80	18% casein " "	41
1654♂	83	18% edestin " "	38
1655♂	79	18% casein " "	38
1592♂	83	18% " " "	42
1599♂	90	18% " " "	46
1619♀	83	18% " " "	43
1636♀	87	18% " " "	45

<sup>9</sup> F. G. Hopkins: *Jour. Physiol.*, xliv, p. 442, 1912.

<sup>10</sup> M. Ruhner: *Die Gesetze des Energieverbrauchs bei Ernährung*, Leipzig, 1902, p. 83, quoted by G. Lusk: *The Elements of the Science of Nutrition*, 2d edition, Philadelphia, 1909, p. 218.

<sup>11</sup> The figures are given in gm. rather than estimated calories, because the mixtures are essentially alike in their physiological fuel value. Protein is replaced by starch or sugar having approximately equivalent energy values. The "protein-free milk" contains additional protein equal to 0.6 per cent of the food.

*Food intake of rats—Concluded.*

RAT	BODY WEIGHT	NATURE OF FOOD	FOOD INTAKE
	gm.		gm.
<i>Rats failing to grow.</i>			
2104 ♀	88	2% lactalbumin + protein-free milk + butter-fat	52
2112 ♀	88	2% " " " "	47
2185 ♀	80	2% " " " "	33
2202 ♂	80	2% " " " "	36
2292 ♂	76	2% " " " "	55
2437 ♀	79	2% casein " " " "	41
2450 ♀	82	2% " " " "	47
2429 ♀	78	2% edestin " " " "	40
2462 ♀	80	2% " " " "	42
2430 ♀	81	2% glutenin " " " "	41
2446 ♀	81	2% " " " "	38
2432 ♀	83	2% glycinin " " " "	39
2461 ♀	79	2% " " " "	40
2435 ♀	80	2% gliadin " " " "	29
2463 ♀	85	2% " " " "	45
2428 ♀	75	No protein " " " "	38
2445 ♀	78	" " " "	33

*Comparative efficiency of proteins for growth and maintenance.*

By the methods employed it is possible to compare the efficiency of different proteins in promoting growth or maintenance. With an abundance of protein in the diet, along with suitable non-protein adjuvants, adequate growth has been observed with the most diverse proteins, such as:

<i>Proteins of animal origin</i>	<i>Proteins of vegetable origin</i>
Casein (milk) <sup>12, 13, 14, 15, 16, 17, 18</sup>	Edestin (hemp-seed) <sup>12, 13, 14, 15, 16, 17, 18</sup>
Lactalbumin (milk) <sup>12, 13, 16, 18</sup>	Globulin (squash-seed) <sup>13, 16</sup>
Ovalbumin (hen's egg) <sup>13, 16, 17</sup>	Excelsin (Brazil nut) <sup>13</sup>
Ovovitelin (hen's egg) <sup>13, 16</sup>	Glutelin (maize) <sup>13, 16, 19</sup>
	Globulin (cottonseed) <sup>13</sup>
	Glutenin (wheat) <sup>12, 13, 15, 16</sup>
	Glycinin (soy bean) <sup>12, 13</sup>
	Glutelin (hemp-seed) <sup>13</sup>

<sup>12</sup> Osborne and Mendel: Feeding Experiments with Isolated Food-Substances, *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911.

(See footnotes on page 362.)

It is interesting to contrast not only the comparative rate of growth with different concentrations of the same protein (see Chart I for casein, Chart II for edestin, Chart III for lactalbumin), but also the unlike ability to grow on diets containing the same percentage of different proteins (Charts VI and VII). Criticisms which may be applied to this method of comparison will be discussed later.

Closely related to these features of nutrition are the *comparative* results obtained with the same concentration of different proteins when the nitrogenous intake is kept below the level at which adequate maintenance is possible. Even here the gradual fall of body weight is *unlike* with the different proteins (see Chart VIII). For further comparison Rats 2428 and 2445 were fed on diets otherwise similar, but containing only the exceptionally small amount of protein (0.63 per cent) present in the "protein-free milk."

*The problem of the protein minimum.* In studying the much debated question of the protein minimum animals have usually been fed with varying quantities of the mixed proteins characteristic of familiar food products. The foregoing discussion of our results with diets containing a *single* protein naturally suggests that the inequalities of the albuminous compounds in respect to their amino-acid make-up will give them quite unlike values when the minimum quantities for maintenance or for growth are approached, the energy intake remaining the same. To cite an extreme case, no amount of zein food, however large, will enable rats to maintain their nutritive equilibrium. A small addition of tryptophane will at once convert the inefficient food into a maintenance ration. A rat has been kept without change of weight for more than six months on a diet containing zein and tryptophane as the sources of nitrogen (see Chart IX).<sup>20</sup> If

<sup>13</sup> Osborne and Mendel: *Ztschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

<sup>14</sup> Osborne and Mendel: *this Journal*, xii, p. 81, 1912.

<sup>15</sup> Osborne and Mendel: *ibid.*, xii, p. 473, 1912.

<sup>16</sup> Osborne and Mendel: *ibid.*, xv, p. 311, 1913.

<sup>17</sup> Osborne and Mendel: *ibid.*, xvi, p. 423, 1913-14.

<sup>18</sup> Osborne and Mendel: *ibid.*, xvii, p. 401, 1914.

<sup>19</sup> Osborne and Mendel: *ibid.*, xviii, p. 1, 1914.

<sup>20</sup> For other data of this sort see Osborne and Mendel: *ibid.*, xvii, p. 325, 1914.

a single amino-acid, tryptophane, can play a rôle thus important in maintenance, it is more than likely that as the lowest limits of requirement are approached the inequalities in the proteins will make themselves noticeable in the unlike quantities needed for the different physiological performances. For example, with the other essential amino-acids equally well provided, the required minimum of the protein lowest in its yield of tryptophane may be expected to be greater than that of a protein comparatively rich in tryptophane precursors.

The inequalities of different sources of protein in meeting the nutritive needs have been recognized in recent years by various investigators. In considering the comparative nutritive values of different isolated proteins one is at once confronted with the difficulty of comparing the nitrogenous needs of different animals of different sizes and sexes at different ages. It is generally believed that in growth a liberal supply of protein is required for constructive purposes beside what the wear-and-tear functions call for. For the present we need not consider the added uses of protein as a physiological fuel material. How is the protein requirement of animals of unlike size to be measured? What unit shall serve as a basis for comparison? Evidently an older adult with an abundance of reserve fat cannot be contrasted gram per gram of body weight with a poorly nourished adolescent animal.

In the experiments in this direction which we have thus far conducted the animals have had access to unlimited quantities of the mixtures of isolated food substances under investigation. The amount of food eaten, *i.e.*, the total energy intake, has now been ascertained in a large number of instances of normal growth on such rations. The word "normal" is here used as synonymous with the average rate or curve of growth exhibited by the same species and sex living on unlimited quantities of a suitable mixed diet. To enable the reader to appreciate the range of variations in this matter the grams of food eaten per week by rats which were making normal growth have been ascertained at different stages of growth on the comparable diets given in the tables on pages 365 and 366. In each group the foods of the individual animals differ in no respect except with regard to the protein, which is casein in some trials and edestin in others.

The averages are expressed in graphic form in Chart X of the



appendix. The early consistently smaller rate of food intake of normally growing rats on diets containing butter-fat, of which the remarkable potency in facilitating growth has been discussed elsewhere,<sup>21</sup> is perhaps to be expected; for if a food is deficient in some essential element, it seems reasonable to suppose that the animal will endeavor to remedy the deficiency by increasing its food intake up to the point where excess of food above the calorific and other requirements of the animal will bring about the well known disturbances of digestion caused by over-eating, and consequently put an end to further increase of food intake.

The range of variation in the tabulated results on these strictly comparable diets is not inconsiderable; yet when one considers differences in the muscular activity of the different individuals, they may after all not exceed what this variable factor would account for. At present this must remain mere conjecture. The ideal method of ascertaining the protein minimum would consist in feeding exactly equivalent amounts of energy in the form of foods with unlike proportions of the individual proteins, thereby learning from the failure of proper gains where the minimum for growth lies, or what the limit of intake for maintenance consists in, when that is the function under consideration. Until recently it has been impracticable, if not impossible, for us to follow this procedure, although we now have experiments relating to this problem in progress.

In our observations on the rate of growth as well as the efficiency of maintenance when rats were fed on food mixtures containing a different content of protein, ranging from 18 per cent (which sufficed for adequate growth) to 2 per cent (which has not sufficed for maintenance even) (Charts I, II, III, VI, VII, VIII), the rate of food intake shows that the failures cannot usually be explained by a lack of energy in the diet. Frequently rats which failed to grow normally were eating sufficient food to enable them to grow if the protein factor had been adequate.<sup>22</sup> In many cases the food intake exceeded the average of those given in the

<sup>21</sup> Osborne and Mendel: *ibid.*, xvi, p. 423, 1913; xvii, p. 401, 1914. E. V. McCollum and M. Davis: *ibid.*, xv, p. 167, 1913.

<sup>22</sup> For a discussion of the relation of growth to food intake, compare Hopkins: *Jour. Physiol.*, xlv, p. 425, 1912.

*Rate in grams of food intake per week of male rats fed with edestin or casein plus protein-free milk food, without butter-fat.*

		BODY WEIGHT OF RAT IN GRAMS																	
		50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220
	31	44	47	52	57	57	57	65	69	75	77	76	75	80	81	85	83	81	76
	29	36	45	51	53	53	57	60	67	69	74	74	75	79	81	84	81	78	75
	26	36	44	50	53	53	56	60	65	69	74	74	75	77	81	82	78	78	72
		35	41	49	53	55	55	59	65	68	71	72	75	77	80	81	76	74	69
		34	41	47	52	54	54	58	64	66	70	73	74	77	76	77	74	72	
		33	40	47	52	54	54	57	63	65	70	71	73	77	75	77	74	70	
		33	40	46	52	54	54	57	61	65	69	71	73	76	74	74	68	68	
		33	40	46	51	54	54	57	61	65	68	69	73	75	74	73	66	66	
		32	40	45	51	53	53	57	61	65	68	69	72	74	74	74	73	65	
		38	45	50	53	56	56	60	64	68	68	68	71	73	73	71			
		38	45	49	53	56	56	59	63	67	68	71	73	72	73	68			
		37	44	49	53	56	56	59	63	66	66	68	71	72	70	68			
		36	44	47	53	56	56	59	63	64	66	66	69	70	68				
		36	44	47	51	55	55	59	62	64	65	65	69	69	67				
		35	43	47	51	55	55	59	61	63	63	65	67	68	66				
		43	47	51	54	58	58	61	63	65	65	66	68	68	66				
		42	45	50	54	57	57	61	63	65	65	66	68	68	66				
		41	45	50	54	56	56	59	62	63	63	65	65	67	63				
		40	45	50	53	56	56	59	62	64	65	65	66	67					
		38	43	48	53	57	57	60	63	65	65	66	68	68					
		35	40	45	50	53	53	56	60	63	65	65	66	68					
		35	40	45	50	53	53	56	60	63	65	65	66	68					
		48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48
		44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44
Average	28.7	35	40	45	49	52	55	59	62.6	65	66	68.7	72	73	75	74	73.5	73	

Rate in grams of food intake per week of male rats fed with casein or edestin plus protein-free milk plus butter or butter-fat food.																										
BODY WEIGHT OF RAT IN GRAMS																										
	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	
	35	47	48	50	52	57	58	62	62	69	77	76	77	82	83	95	104	104	102	93	83	84	94	100	96	
	34	38	43	48	52	56	56	59	61	64	67	70	73	75	80	87	88	84	91	85	82	83	92	95	88	
	32	38	42	47	49	51	54	58	60	62	66	68	70	73	75	81	84	83	85	81	81	83	91	86	84	
	27	37	41	47	47	50	54	57	59	61	63	67	69	72	74	79	82	80	83	79	81	82	84	85	83	
		35	41	44	44	49	52	56	59	60	62	67	68	71	74	77	74	78	76	76	79	82	84	81	81	
		35	41	42	43	48	51	55	57	60	62	66	67	71	73	77	74	75	73	75	76	80	72	78		
		33	40	41	43	47	50	54	57	60	62	66	67	69	72	73	72	69	70	72						
			39	40	43	46	50	54	56	59	61	65	66	67	71	71	71	68	69							
			38	40	41	45	50	53	55	58	60	59	66	67	65	68	62	65								
			36	40	40	43	48	52	54	55	54	58	65	61												
			34	38	40	42	47	49	52	54	54	57	64													
				36	38	41	44	47	51	53																
Average.....	32	37.6	40	43	44	48	51	55	57	59.6	62.5	65	68	70.8	74	78.7	79	78.81	80	80	80	82	86	87	86	

table on page 366 on which a normal growth was being made. These are not isolated instances. They have been repeated again and again and answer the obvious criticism that the failure to grow properly was always due to a deficiency in ingested calories.

With our mode of investigation and its not inconsiderable limitations thus outlined, it is of interest, as has already been pointed out, to compare the *relative* efficiency of the same concentrations of different proteins. Charts VI, VII, and VIII present results of this sort. The unlike efficiency of 12, 9, 6, 4.5, and 2 per cent of the proteins casein, edestin, and lactalbumin, is at once apparent. A content of protein which is utterly inadequate in the case of casein permits good growth when lactalbumin is used. This, again, is not due to marked differences in the actual absolute consumption of protein, as the following data demonstrate:

RAT	DIET	INITIAL BODY WEIGHT	GAIN		INTAKE OF	
		gm.	gm.	days	Total food gm.	Protein gm.
2115♂.....	9% lactalbumin	65	99	80	589	43.7
2123♂.....	"	43	89	80	395	29.3
2207♂.....	"	59	91	80	575	42.6
2210♀.....	"	60	93	80	560	41.5
2051♂.....	9% casein	42	59	80	435	35.2
2110♀.....	9% edestin	52	60	80	465	38.9
2044♂.....	4½% lactalbumin	42	44	80	371	13.8
2049♀.....	"	41	54	80	454	16.8
2118♂.....	4½% casein	59	-3	80	366	14.8
2113♀.....	4½% edestin	83	14	80	459	19.2
2114♀.....	"	65	29	80	464	19.4

It will be observed that the rats supplied with the 9 per cent casein or edestin food ate quantities of protein approximately similar to those on the 9 per cent lactalbumin food, but gained only two-thirds as much in weight during the same number of days. A comparison of the animals on the foods containing 4.5 per cent of these proteins shows that while the protein intakes were of approximately the same order, the gains on lactalbumin were relatively much greater than in the experiments with the other 4.5 per cent foods.

## 368      Nutritive Value of Proteins in Growth

A more critical comparison of some of the proteins on the basis of their efficiency in permitting maintenance or promoting growth will be possible when further data are available. The facts adduced above correspond with observations already published by us to demonstrate that the proteins have unlike physiological values in supplementing the deficiencies of zein as a dietary protein.<sup>23</sup> The economy of the different proteins as nutrients in growth appears to be closely bound up with their amino-acid make-up.

### APPENDIX.

The composition of the foods used was as follows:<sup>24</sup>

CASEIN											
per cent											
Protein.....	18	15	12	11.64	9	9	6	6	4.5	4.5	2
Cystine.....				0.36		0.54		0.54		0.135	
Protein-free milk.....	28	28	28	28	28	28	28	28	28	28	28
Sucrose.....			7	7	9	9	10	10	10	10	12
Starch.....	29	32	25	18	29	28.46	31	30.46	31.5	31.365	32
Butter-fat.....	18	6-18	18	10	18	6-18	18	18	18	18	18
Lard.....		7,19-7	10			7,19-7	7	7	8	8	8

	LACTALBUMIN					OVAL-BUMIN		OVOVITELLIN		
	per cent					per cent		per cent		
Protein.....	18	11	9	4.5	2.5	2	1	9	9	0
Protein-free milk....	28	28	28	28	28	28	28	28	28	28
Sucrose.....		5	9	10	12	12	12	8	8	11
Starch.....	28	30	29	31.5	31.5	32	33	29	29	27
Butter-fat.....	18	6-18	18	18	18	18	18	6-18	6-18	18
Lard.....		8,20-8	7	8	8	8	8	20-8	20-8	6
Lactose.....										10

<sup>23</sup> Osborne and Mendel: this *Journal*, xvii, p. 325, 1914; xviii, p. 1, 1914.

<sup>24</sup> These figures refer to the quantities of air-dry foodstuffs; in the text the data for protein intake are calculated to a water-free basis.

	MILK PROTEINS				CORN GLUTEN + LACTALBUMIN	
	per cent				per cent	
Protein.....	12	9	6	Protein.....	9	6
Milk powder.....	46	35	25	Corn gluten.....	13	8.8
Protein-free milk.....	8	11	15	Lactalbumin.....	3	2
Starch.....	19	27	33	Protein-free milk.....	28	28
Butter-fat.....	5.5	7	9	Starch.....	28	33.2
Lard.....	21.5	20	18	Butter-fat.....	18	18
				Lard.....	10	10

EDESTIN									
per cent									
Protein.....	18	15	12	11.46-11.76	9	9	4.5	2	
Lysine dichloride.....				0.36-0.81		0.81			
Na <sub>2</sub> CO <sub>3</sub> .....				0.17-0.39		0.39			
Protein-free milk.....	28	28	28	28.00	28	28	28	28	
Sucrose.....			7	7.00	7-9	9	10	12	
Starch.....	22	29	25	21.71-24.34	28-29	28.8	31.5	32	
Butter-fat.....	18	6-18	18	18.00	18	6-18	18	18	
Lard.....	14	22-10	10	10.00	10-7	18-6	8	8	

	GLUTENIN		GLYCININ			SQUASH-SEED GLOBULIN		GLIADIN	
	per cent		per cent			per cent		per cent	
Protein.....	9	2	9	4.5	2	9	4.5	9	2
Protein-free milk....	28	28	28	28	28	28	28	28	28
Sucrose.....	6	12	6	10	12	6	10	7	12
Starch.....	29	32	29	31.5	32	29	31.5	28	32
Butter-fat.....	6	18	6	18	18	6	18	18	18
Lard.....	22	8	22	8	8	22	8	10	8

ZEIN			
gm.			
Protein.....	18	17.46	16.92
Tryptophane.....		0.54	0.54
Lysine dichloride.....			0.81
Na <sub>2</sub> CO <sub>3</sub> .....			0.39
Protein-free milk.....	28	28	28
Starch.....	27	27	26.34
Butter-fat.....	18	18	18
Lard.....	9	9	9
Water.....	15	15	15

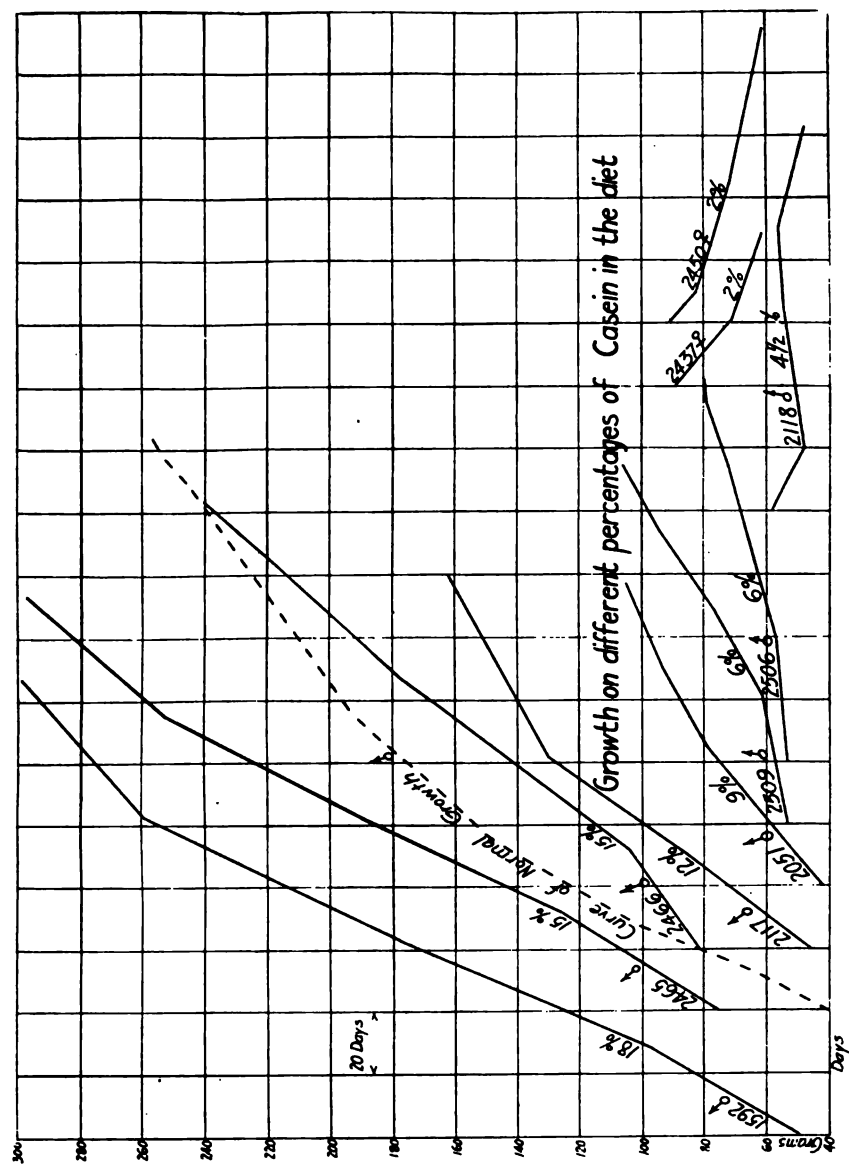


CHART I. Comparison of growth on diets containing different percentages of casein. The composition of the foods used is given in the tables in the appendix.

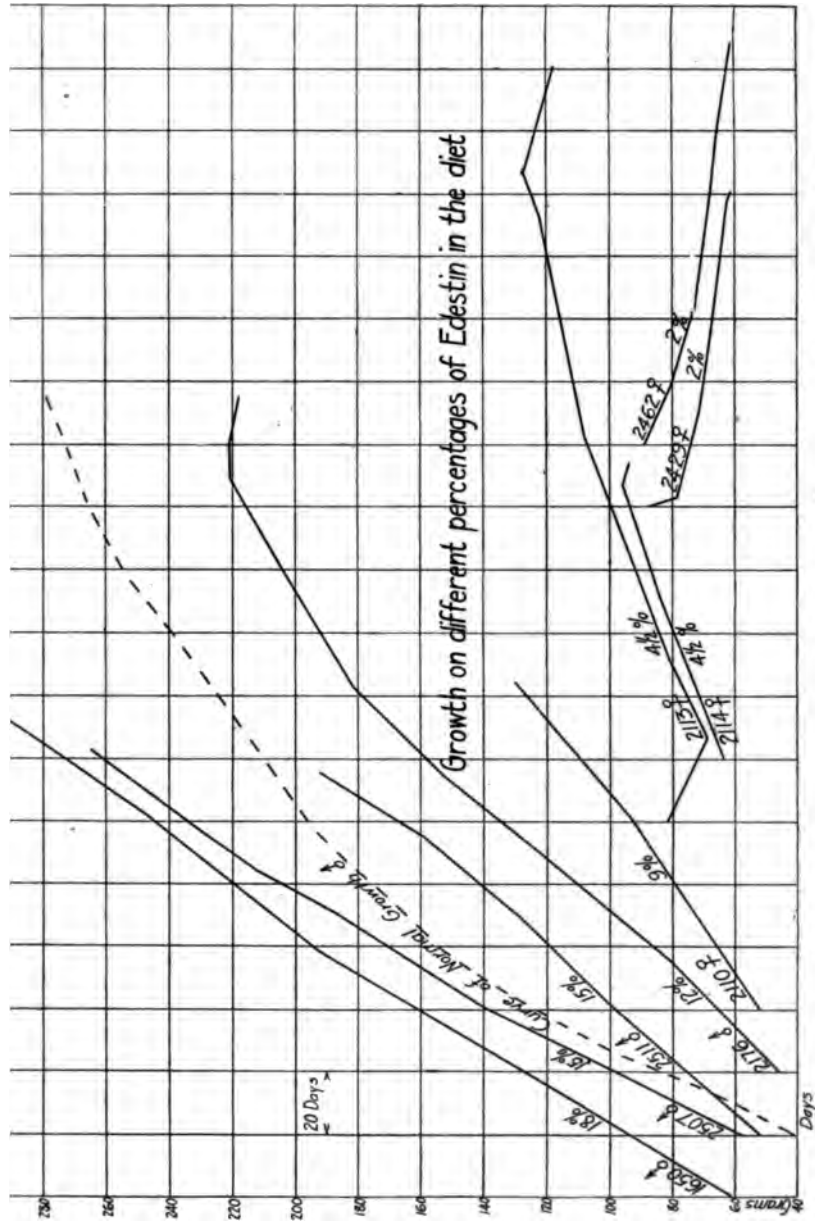


CHART II. Comparison of growth on diets containing different percentages of edestin. The composition of the foods used is given in the tables in the appendix.



It is interesting to contrast not only the comparative rate of growth with different concentrations of the same protein (see Chart I for casein, Chart II for edestin, Chart III for lactalbumin), but also the unlike ability to grow on diets containing the same percentage of different proteins (Charts VI and VII). Criticisms which may be applied to this method of comparison will be discussed later.

Closely related to these features of nutrition are the *comparative* results obtained with the same concentration of different proteins when the nitrogenous intake is kept below the level at which adequate maintenance is possible. Even here the gradual fall of body weight is *unlike* with the different proteins (see Chart VIII). For further comparison Rats 2428 and 2445 were fed on diets otherwise similar, but containing only the exceptionally small amount of protein (0.63 per cent) present in the "protein-free milk."

*The problem of the protein minimum.* In studying the much debated question of the protein minimum animals have usually been fed with varying quantities of the mixed proteins characteristic of familiar food products. The foregoing discussion of our results with diets containing a *single* protein naturally suggests that the inequalities of the albuminous compounds in respect to their amino-acid make-up will give them quite unlike values when the minimum quantities for maintenance or for growth are approached, the energy intake remaining the same. To cite an extreme case, no amount of zein food, however large, will enable rats to maintain their nutritive equilibrium. A small addition of tryptophane will at once convert the inefficient food into a maintenance ration. A rat has been kept without change of weight for more than six months on a diet containing zein and tryptophane as the sources of nitrogen (see Chart IX).<sup>20</sup> If

<sup>13</sup> Osborne and Mendel: *Ztschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

<sup>14</sup> Osborne and Mendel: *this Journal*, xii, p. 81, 1912.

<sup>15</sup> Osborne and Mendel: *ibid.*, xii, p. 473, 1912.

<sup>16</sup> Osborne and Mendel: *ibid.*, xv, p. 311, 1913.

<sup>17</sup> Osborne and Mendel: *ibid.*, xvi, p. 423, 1913-14.

<sup>18</sup> Osborne and Mendel: *ibid.*, xvii, p. 401, 1914.

<sup>19</sup> Osborne and Mendel: *ibid.*, xviii, p. 1, 1914.

<sup>20</sup> For other data of this sort see Osborne and Mendel: *ibid.*, xvii, p. 325, 1914.

a single amino-acid, tryptophane, can play a rôle thus important in maintenance, it is more than likely that as the lowest limits of requirement are approached the inequalities in the proteins will make themselves noticeable in the unlike quantities needed for the different physiological performances. For example, with the other essential amino-acids equally well provided, the required minimum of the protein lowest in its yield of tryptophane may be expected to be greater than that of a protein comparatively rich in tryptophane precursors.

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The averages are expressed in graphic form in Chart X of the

appendix. The early consistently smaller rate of food intake of normally growing rats on diets containing butter-fat, of which the remarkable potency in facilitating growth has been discussed elsewhere,<sup>21</sup> is perhaps to be expected; for if a food is deficient in some essential element, it seems reasonable to suppose that the animal will endeavor to remedy the deficiency by increasing its food intake up to the point where excess of food above the calorific and other requirements of the animal will bring about the well known disturbances of digestion caused by over-eating, and consequently put an end to further increase of food intake.

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BODY WEIGHT OF RAT IN GRAMS																	
50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220
31	44	47	52	57	57	65	69	75	77	76	75	80	81	85	83	81	76
29	36	45	51	53	57	60	67	69	74	74	75	79	81	84	81	78	75
26	36	44	50	53	56	60	65	69	74	74	75	77	81	82	78	78	72
	35	41	49	53	55	59	65	68	71	72	75	77	80	81	76	74	69
	34	41	47	52	54	58	64	66	70	73	74	77	76	77	74	72	
	33	40	47	52	54	57	63	65	70	71	73	77	75	77	74	70	
	33	40	46	52	54	57	61	65	69	71	73	76	74	74	68	68	
	33	40	46	51	54	57	61	65	68	69	73	75	74	73	66	66	
	32	40	45	51	53	56	61	65	68	69	72	74	74	73	65		
		38	45	50	53	56	60	64	68	68	71	73	73	71			
		38	45	49	53	56	59	63	67	68	71	72	73	68			
		37	44	49	53	56	59	63	66	68	71	72	70	68			
		36	44	47	53	56	59	63	64	66	70	71	70	62			
		36	44	47	51	55	59	62	64	66	69	69	67				
		35	43	47	51	55	59	61	63	65	69	69	68				
		43	43	47	51	54	58	61	63	65	67	68	66				
		43	43	47	50	54	57	61	63	65	66	68	66				
		42	45	45	50	54	56	58	62	63	65	67	63				
		41	45	45	50	53	56	57	61	62	65	67					
		40	45	49	53	55	57	57	59	60	63	66					
		38	43	49	53	54	57	57	59	59	62	63					
		35	40	48	50	53	55	55	58	58	60						
			48	48	48	48	53	52	57	58	59						
			44	44	44	44	49	49	52	56	54						
							46										
Average	28.7	35	40	45	49	52	55	59	62.6	65	66	68.7	72	73	75	74	73

Rate in grams of food intake per week of male rats fed with casein or edestin plus protein-free milk plus butter or butter-fat food.																									
BODY WEIGHT OF RAT IN GRAMS																									
60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	
35	47	48	50	52	57	58	62	62	69	77	76	77	82	83	95	104	104	102	93	83	84	94	100	96	
34	38	43	48	52	56	56	59	61	64	67	70	73	75	80	87	88	84	91	85	82	83	92	95	88	
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27	37	41	47	47	50	54	57	59	61	63	67	69	72	74	79	82	80	83	79	81	82	84	85	83	
	35	41	44	44	49	52	56	58	60	62	67	68	71	74	77	74	78	76	76	79	82	84	81	81	
	35	41	42	43	48	51	55	57	60	62	66	67	71	73	77	74	75	73	75	76	80	72	78		
	33	40	41	43	47	50	54	57	60	62	66	67	69	72	73	72	69	70	72						
		39	40	43	46	50	54	56	59	61	65	66	67	71	71	71	68	69							
		38	40	41	45	50	53	55	58	60	59	66	67	65	68	62	65								
		36	40	40	43	48	52	54	55	54	58	65	61												
		34	38	40	42	47	49	52	54	54	57	64													
			36	38	41	44	47	51	53																
32	37.6	40	43	44	48	51	55	57	59.6	62.5	65	68	70.8	74	78.7	79	78	81	80	80	82	86	87	80	
Average.....																									

table on page 366 on which a normal growth was being made. These are not isolated instances. They have been repeated again and again and answer the obvious criticism that the failure to grow properly was always due to a deficiency in ingested calories.

With our mode of investigation and its not inconsiderable limitations thus outlined, it is of interest, as has already been pointed out, to compare the *relative* efficiency of the same concentrations of different proteins. Charts VI, VII, and VIII present results of this sort. The unlike efficiency of 12, 9, 6, 4.5, and 2 per cent of the proteins casein, edestin, and lactalbumin, is at once apparent. A content of protein which is utterly inadequate in the case of casein permits good growth when lactalbumin is used. This, again, is not due to marked differences in the actual absolute consumption of protein, as the following data demonstrate:

RAT	DIET	INITIAL BODY WEIGHT	GAIN			INTAKE OF	
						Total food	Protein
		gm.	gm.	dys.		gm.	gm.
2115♂.....	9% lactalbumin	65	99	80		589	43.7
2123♂.....	"	43	89	80		395	29.3
2207♂.....	"	59	91	80		575	42.6
2210♀.....	"	60	93	80		560	41.5
2051♂.....	9% casein	42	59	80		435	35.2
2110♀.....	9% edestin	52	60	80		465	38.9
2044♂.....	4½% lactalbumin	42	44	80		371	13.8
2049♀.....	"	41	54	80		454	16.8
2118♂.....	4½% casein	59	-3	80		366	14.8
2113♀.....	4½% edestin	83	14	80		459	19.2
2114♀.....	"	65	29	80		464	19.4

It will be observed that the rats supplied with the 9 per cent casein or edestin food ate quantities of protein approximately similar to those on the 9 per cent lactalbumin food, but gained only two-thirds as much in weight during the same number of days. A comparison of the animals on the foods containing 4.5 per cent of these proteins shows that while the protein intakes were of approximately the same order, the gains on lactalbumin were relatively much greater than in the experiments with the other 4.5 per cent foods.

## 368      Nutritive Value of Proteins in Growth

A more critical comparison of some of the proteins on the basis of their efficiency in permitting maintenance or promoting growth will be possible when further data are available. The facts adduced above correspond with observations already published by us to demonstrate that the proteins have unlike physiological values in supplementing the deficiencies of zein as a dietary protein.<sup>23</sup> The economy of the different proteins as nutrients in growth appears to be closely bound up with their amino-acid make-up.

### APPENDIX.

The composition of the foods used was as follows:<sup>24</sup>

CASEIN												
per cent												
Protein.....	18	15	12	11.64	9	9	6	6	4.5	4.5	2	
Cystine.....				0.36		0.54		0.54		0.135		
Protein-free milk.....	28	28	28	28	28	28	28	28	28	28	28	
Sucrose.....			7	7	9	9	10	10	10	10	12	
Starch.....	29	32	25	18	29	28.46	31	30.46	31.5	31.365	32	
Butter-fat.....	18	6-18	18	10	18	6-18	18	18	18	18	18	
Lard.....		7-19-7	10			7-19-7	7	7	8	8	8	

LACTALBUMIN												
per cent												
Protein.....	18	11	9	4.5	2.5	2	1	9	9	0		
Protein-free milk.....	28	28	28	28	28	28	28	28	28	28		
Sucrose.....		5	9	10	12	12	12	8	8	11		
Starch.....	28	30	29	31.5	31.5	32	33	29	29	27		
Butter-fat.....	18	6-18	18	18	18	18	18	6-18	6-18	18		
Lard.....		8-20-8	7	8	8	8	8	20-8	20-8	6		
Lactose.....										10		

<sup>23</sup> Osborne and Mendel: this *Journal*, xvii, p. 325, 1914; xviii, p. 1, 1914.

<sup>24</sup> These figures refer to the quantities of air-dry foodstuffs; in the text the data for protein intake are calculated to a water-free basis.

MILK PROTEINS			CORN GLUTEN + LACTALBUMIN		
per cent			per cent		
Protein.....	12	9 6	Protein.....	9	6
Milk powder.....	46	35 25	Corn gluten.....	13	8.8
Protein-free milk.....	8	11 15	Lactalbumin.....	3	2
Starch.....	19	27 33	Protein-free milk.....	28	28
Butter-fat.....	5.5	7 9	Starch.....	28	33.2
Lard.....	21.5	20 18	Butter-fat.....	18	18
			Lard.....	10	10

## EDESTIN

	<i>per cent</i>							
Protein.....	18	15	12	11.46-11.76	9	9	4.5	2
Lysine dichloride.....				0.36-0.81			0.81	
Na <sub>2</sub> CO <sub>3</sub> .....				0.17-0.39			0.39	
Protein-free milk.....	28	28	28	28.00	28	28	28	28
Sucrose.....			7	7.00	7-9	9	10	12
Starch.....	22	29	25	21.71-24.34	28-29	28.8	31.5	32
Butter-fat.....	18	6-18	18	18.00	18	6-18	18	18
Lard.....	14	22-10	10	10.00	10-7	18-6	8	8

	GLUTENIN		GLYCININ			SQUASH-SEED GLOBULIN		GLIADIN	
	per cent		per cent			per cent		per cent	
Protein.....	9	2	9	4.5	2	9	4.5	9	2
Protein-free milk....	28	28	28	28	28	28	28	28	28
Sucrose.....	6	12	6	10	12	6	10	7	12
Starch.....	29	32	29	31.5	32	29	31.5	28	32
Butter-fat.....	6	18	6	18	18	6	18	18	18
Lard.....	22	8	22	8	8	22	8	10	8

## ZEIN

	<i>gm.</i>		
Protein.....	18	17.46	16.92
Tryptophane.....		0.54	0.54
Lysine dichloride.....			0.81
Na <sub>2</sub> CO <sub>3</sub> .....			0.39
Protein-free milk.....	28	28	28
Starch.....	27	27	26.34
Butter-fat.....	18	18	18
Lard.....	9	9	9
Water.....	15	15	15



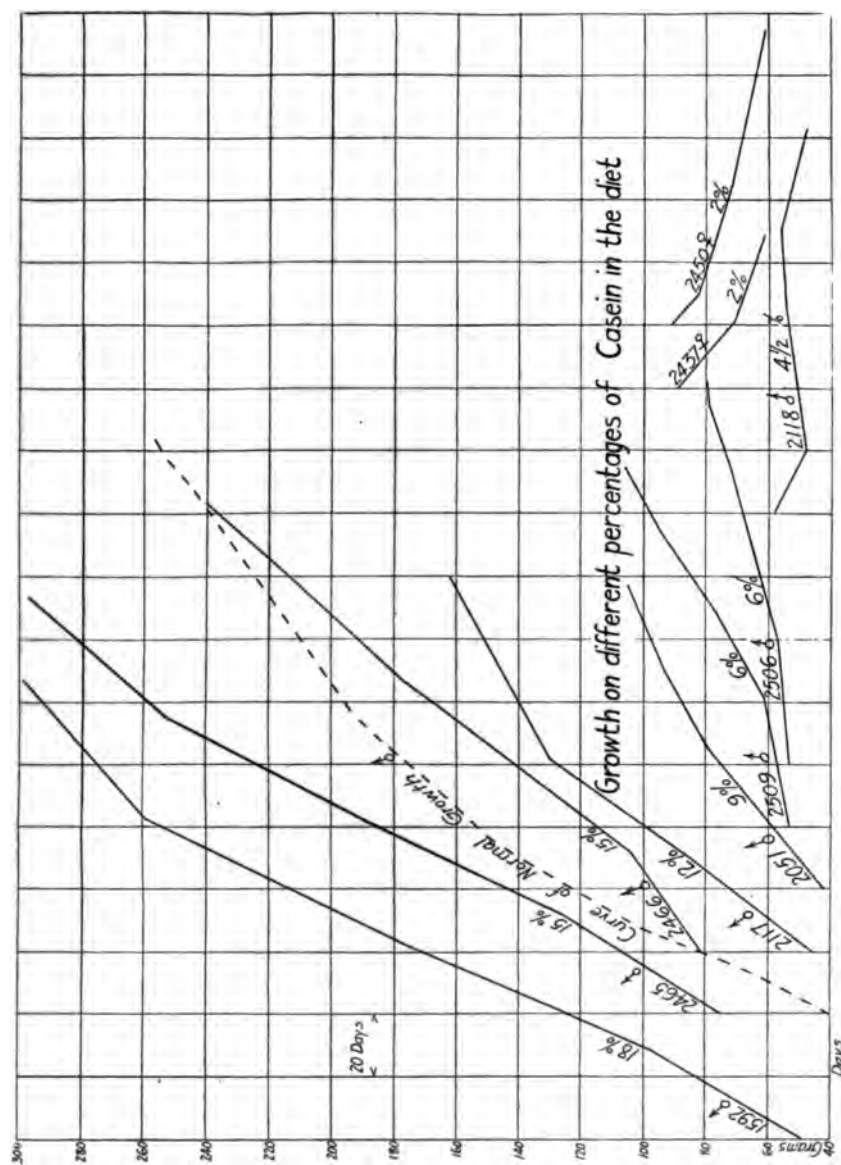


CHART I. Comparison of growth on diets containing different percentages of casein. The composition of the foods used is given in the tables in the appendix.

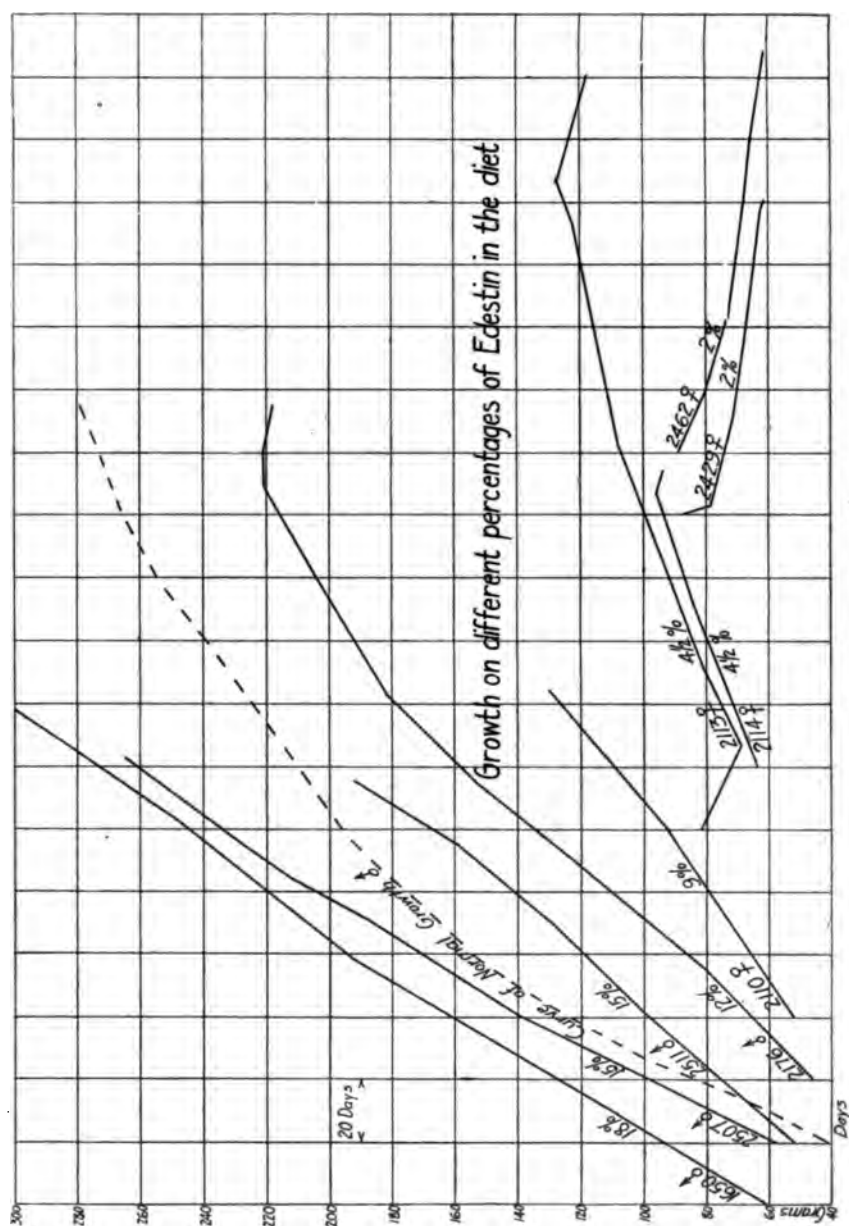


CHART II. Comparison of growth on diets containing different percentages of *edestin*. The composition of the foods used is given in the tables in the appendix.

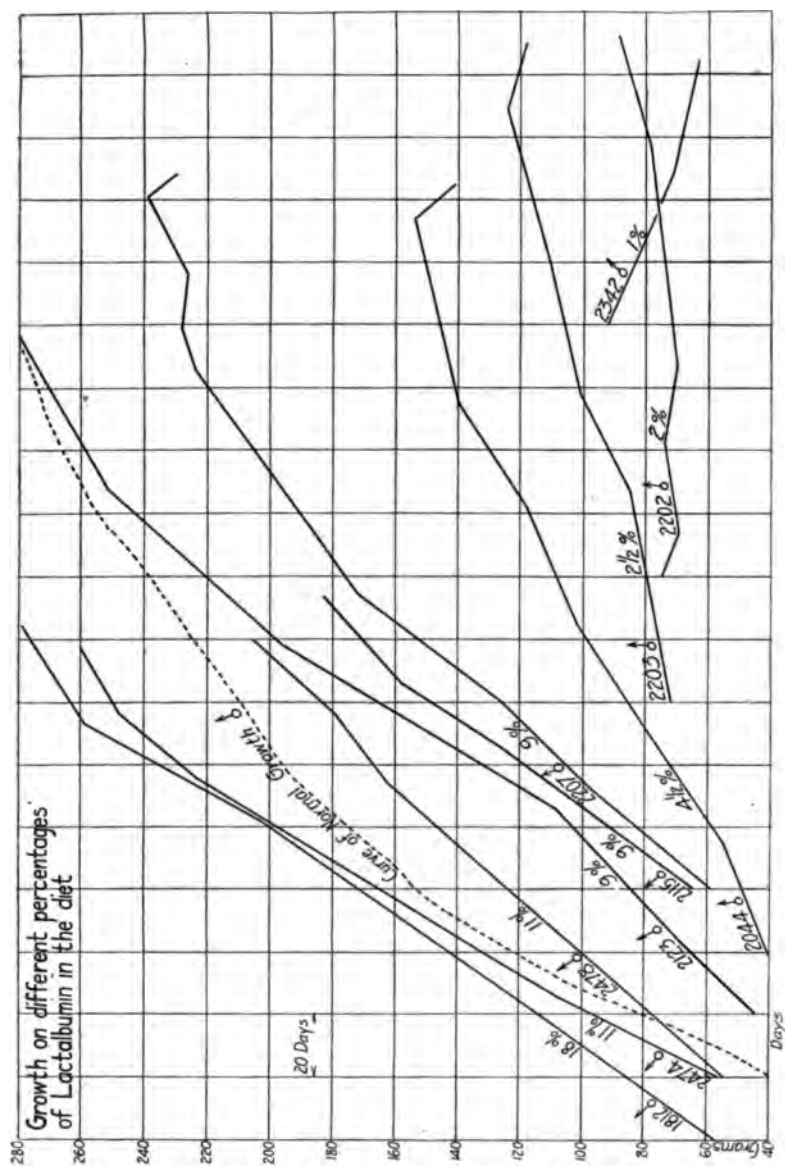


CHART III. Comparison of growth on diets containing different percentages of *lactalbumin*. The composition of the foods used is given in the tables in the appendix.

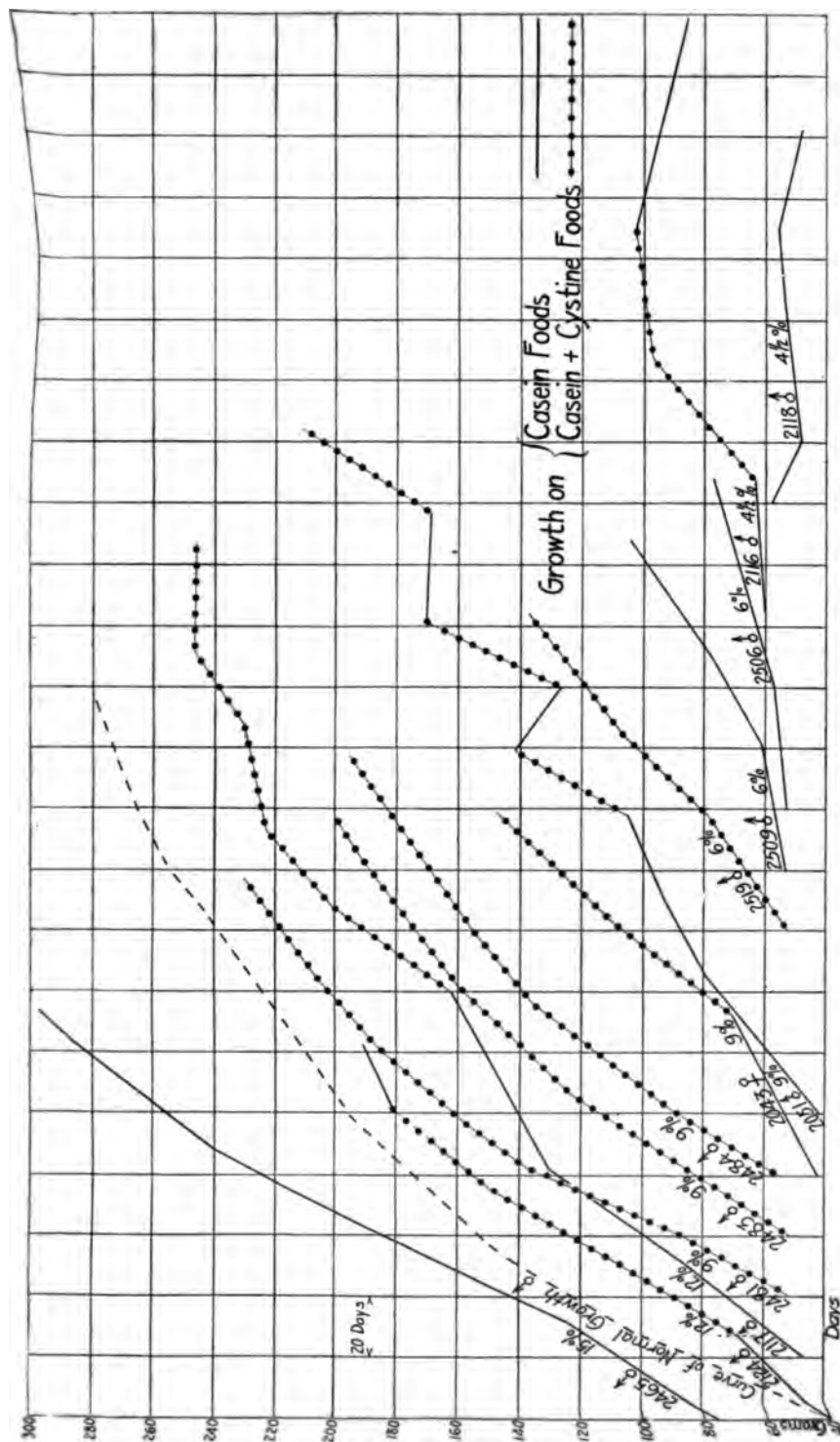


CHART IV. Showing the efficiency of *cystine* in supplementing diets containing percentages of casein insufficient for perfect growth. For comparison the effect of a larger percentage (15 per cent) of casein alone is shown. The comparative deficiency of cystine precursors in casein does not manifest itself until the diet contains less than 15 per cent of the protein. The composition of the foods used is given

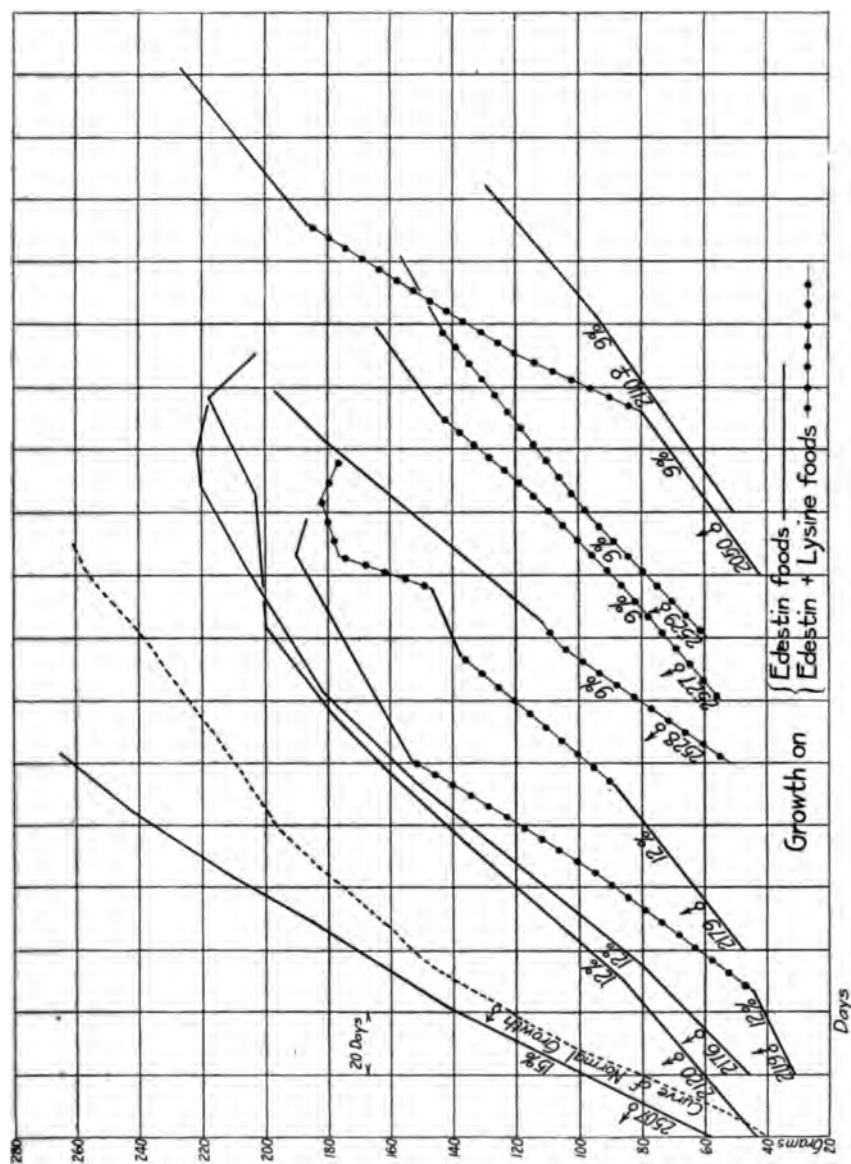


CHART V. Showing the efficiency of *lysine* in supplementing diets containing percentages of edestin insufficient for perfect growth. For comparison the effect of a larger percentage (15 per cent) of edestin alone is shown. The comparative deficiency of lysine precursors in edestin does not manifest itself until the diet contains less than 15 per cent of the protein. The composition of the foods used is given in the tables in the

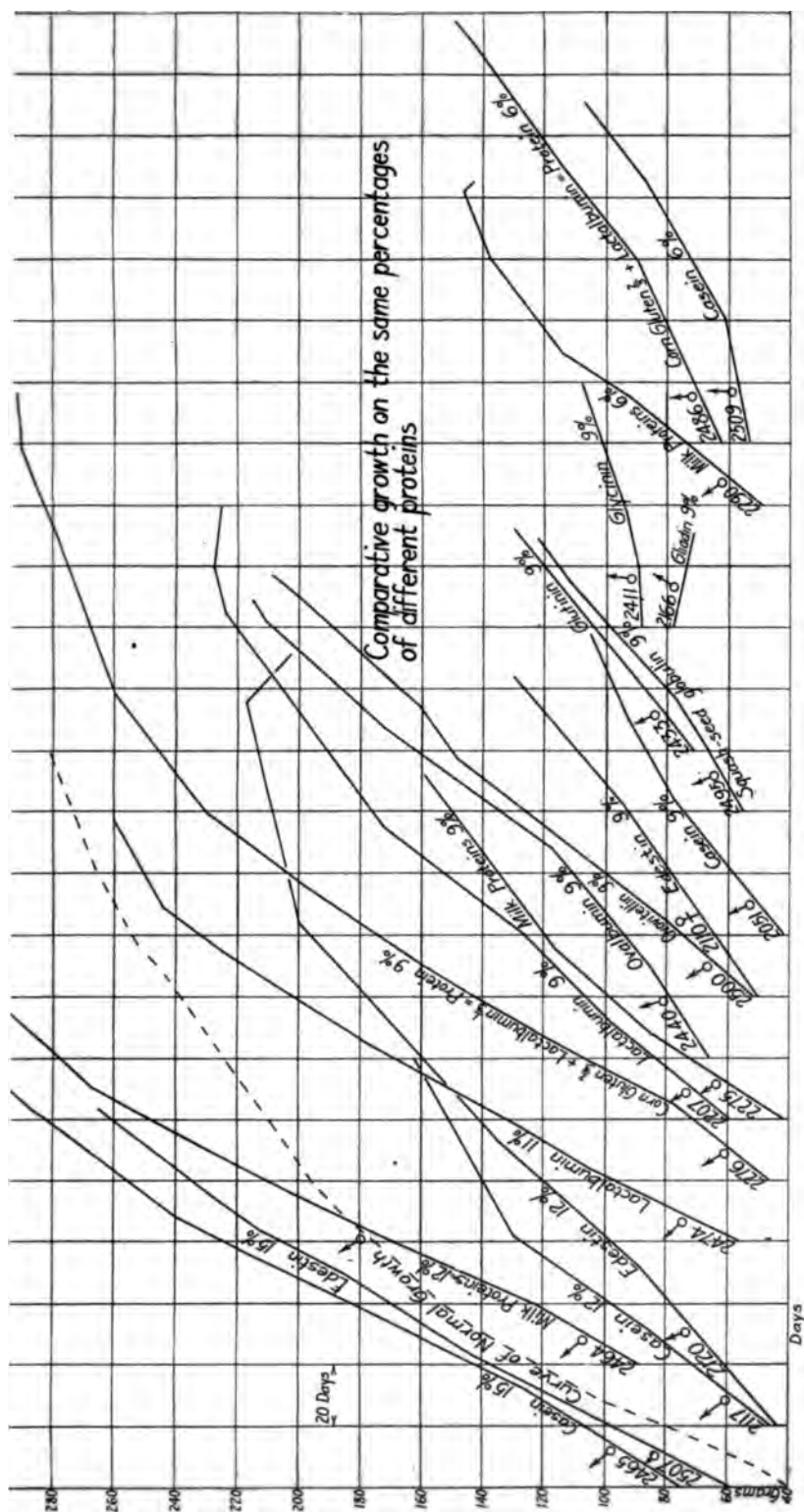


CHART VI. Comparison of growth on diets containing approximately the same percentages of different proteins, namely, 15 per cent of casein and edestin; 12 per cent of milk proteins, casein, edestin; 11 per cent of lactalbumin; 9 per cent of corn gluten + lactalbumin, lactalbumin, milk proteins, oralbumin, edestin, casein, glutenin (wheat), globulin (squash-seed), glycine (soy bean), gliadin (wheat); 6 per cent of milk proteins, corn gluten + lactalbumin, and casein. The composition of the foods used is given in the tables in the appendix.

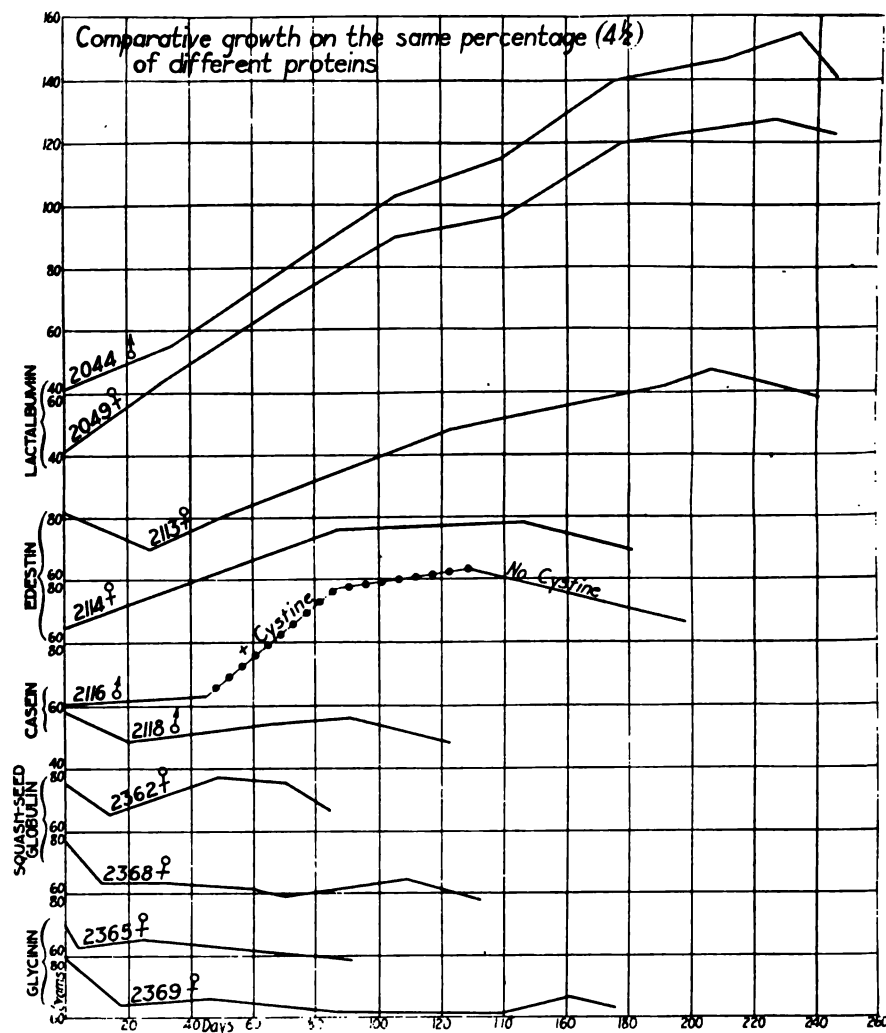


CHART VII. Comparison of growth on diets containing approximately the same percentage (4.5 per cent) of different proteins; namely, *lactalbumin*, *edestin*, *casein*, *globulin* (squash-seed), and *glycinin* (soy bean). The composition of the foods used is given in the tables in the appendix.

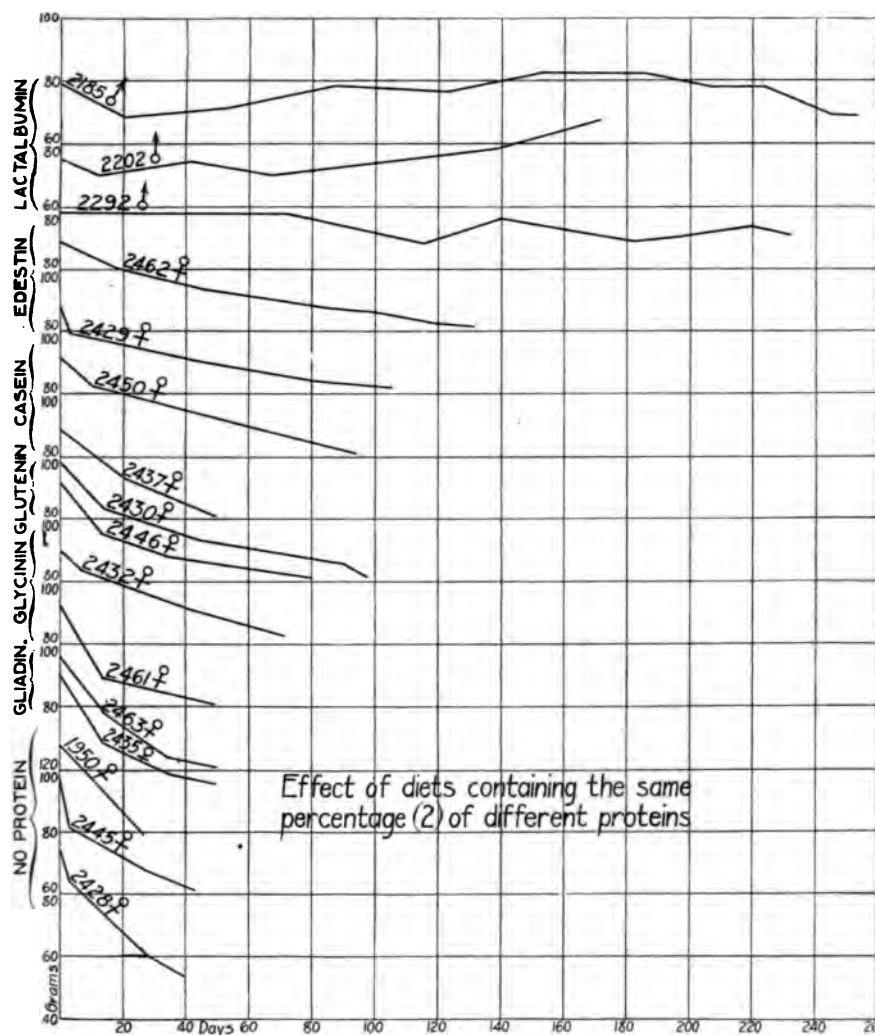


CHART VIII. Comparison of changes in body weight induced by approximately the same inadequate percentage (2 per cent) of *lactalbumin*, *edestin*, *casein*, *glutenin* (wheat), *glycinin* (soy bean), and *gliadin* (wheat) in the diet. For contrast the effect of a practically protein-free diet is shown in Rats 1950, 2445, and 2428. The composition of the foods used is given in the tables in the appendix.



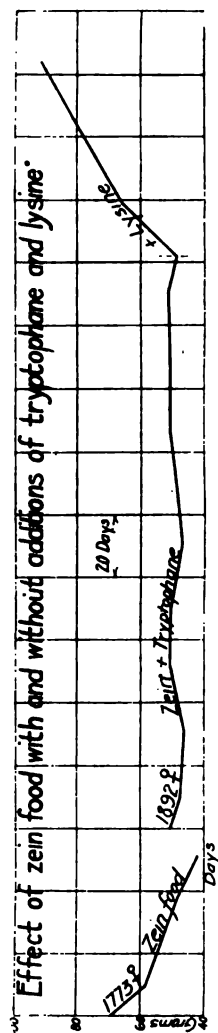


CHART IX. Showing nutritive decline on zein food, maintenance after addition of tryptophane, growth after addition of both tryptophane and lysine. Rat 1892 was maintained during one-half year without significant change in body weight on the zein + tryptophane food. Despite this inadequate diet the capacity to grow was not lost at the end of this prolonged period, and the animal ultimately grew to full adult size on a mixed diet. The exact composition of the foods used is given in the tables in the appendix.

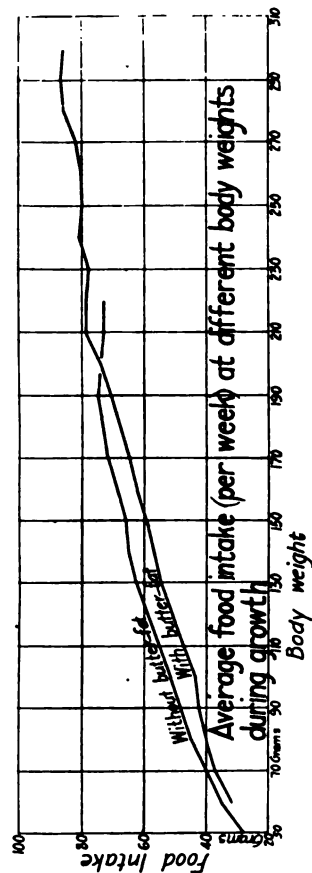


CHART X. Average food intake (per week) at different body weights during growth. The unlike results obtained by the use of different fats in the foods is discussed in connection with the data on pages 365 and 366, from which this chart has been compiled.

## FURTHER OBSERVATIONS OF THE INFLUENCE OF NATURAL FATS UPON GROWTH.<sup>1</sup>

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WITH THE COÖPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

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(Received for publication, January 30, 1915.)

When young albino rats are fed on a ration composed of isolated and purified protein, a carbohydrate like starch, inorganic salts furnished in the form of what we have termed "protein-free milk"<sup>2</sup> (which is likewise fat-free), together with commercial lard, they usually grow normally for about three months, but never attain their full size. Sooner or later nutritive disaster manifests itself by a complete or partial cessation of growth which ultimately (and sometimes precipitately) ends in a decline in body weight, followed by death if a suitable change in diet is not promptly instituted. The disturbance of growth may be attended by symptoms of malnutrition (such as infections of the eye) giving evidence of impaired resistance to bacterial invasion. Recovery promptly follows the substitution of a part of the lard of the ration by certain other natural fats.<sup>3</sup>

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> T. B. Osborne and L. B. Mendel: Feeding Experiments with Isolated Food-Substances, *Carnegie Institution of Washington, Publication No. 156*, pt. ii, p. 80, 1911; see also *Ztschr. f. physiol. Chem.*, lxxx, pp. 315-16, 1912.

<sup>3</sup> E. V. McCollum and M. Davis: *this Journal*, xv, p. 167, 1913. Osborne and Mendel: *ibid.*, xv, p. 311; xvi, p. 423, 1913-14. McCollum and Davis: *Proc. Soc. Exper. Biol. and Med.*, xi, p. 101, 1914. Osborne and Mendel: *this Journal*, xvii, p. 401, 1914. McCollum and Davis: *ibid.*, xix, p. 245, 1914.

The word "fat" is used in this paper to refer to the mixtures which ordinarily are included by that designation, rather than to the pure glycerides of fatty acids.

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The importance of this feature of the dietary in the use of mixtures of isolated food substances as the exclusive ration of growing animals, is emphasized by experience which we have accumulated during several years. Rats have been raised and bred, into the third generation, on diets consisting of:

	<i>per cent</i>
Edestin.....	18
Starch.....	24
"Protein-free milk".....	28
Commercial lard.....	12
Butter-fat.....	18

Some of our animals are still living after 620 days on such a ration. In other words, when the fat component of the diet is suitable from the outset, the peculiar nutritive failures described do not put in their appearance. This lends confirmation to the conclusion that the "standard" diets containing lard or certain other substances as the sole fat admixture are unquestionably deficient in some respect.

The difference in the effects on growth which characterize the different natural fats investigated suggested that perhaps the inefficiency of the lard was due to the technical processes to which the fat of the pigs was subjected in preparation for the market. Lard was therefore prepared in the laboratory by the following procedure from fat brought directly from the slaughter house:

The crude material was comminuted, and filtered through paper at a temperature just above its melting point. Overheating was prevented by putting the lard in a large filter placed in an oven at a temperature just sufficient to melt the fat and allow it to pass slowly through the paper.

With this "laboratory lard," used in place of the commercial product in the diet, without other fats, the cessation of growth occurred quite as promptly and characteristically as before (see Chart I); and restoration was accomplished by the usual method of adding another natural fat (such as butter-fat) to the diet to replace part of the lard. Other facts are in further harmony with the conclusion that the inefficiency of the lard is not due to the heating to which the fat may have been subjected in its preparation. Almond oil, likewise, which failed to restore growth

after the declines on lard diets,<sup>4</sup> was prepared in the laboratory without the aid of heat, by grinding the nuts, pressing out, and filtering the oil at room temperature. On the other hand, butter-fat through which live steam was passed for two and one-half hours or longer did not lose its characteristic restorative properties (see Chart I, Rats 1962 and 1976). McCollum and Davis have recently obtained results indicating that the substance, or substances, present in butter-fat which exert such a marked stimulating action on growth, are sufficiently stable to withstand conditions of saponification which they have employed.<sup>5</sup> Accordingly, certain fats may retain their efficiency in this regard despite vigorous treatment with heat<sup>6</sup> or chemical agents; whereas other fats remain practically inefficient even after the most gentle manipulation.

To the list of natural fats found to contain the factor efficient in facilitating growth on the diet outlined above we can now add beef-fat. The material was prepared as follows:

Abdominal fat from cattle was cut up, and filtered through paper at a temperature just above its melting point. Higher temperatures were avoided by conducting the filtration as with lard (see page 380).

In Chart II are reproduced some of the growth curves of rats fed on a ration of:

	per cent	
Casein.....	18	
Edestin.....		18
Starch.....	29	20
"Protein-free milk".....	28	28
Commercial lard.....	7	16
Beef-fat.....	18	18

In some cases the decline invariably experienced in about three months when lard alone was used has been averted by admixture of *beef-fat*. The recoveries when beef-fat is fed after decline on the lard diets (Rats 1914, 2130, 1924, Chart II), and the continuance of growth when beef-fat feeding is begun very early, are not as marked or prolonged as are those accomplished by butter-fat

<sup>4</sup> Osborne and Mendel: this *Journal*, xvii, p. 401, 1914.

<sup>5</sup> McCollum and Davis: *ibid.*, xix, p. 245, 1914.

<sup>6</sup> In accord with this, McCollum and Davis (*Proc. Soc. Exper. Biol. and Med.*, xi, p. 101, 1914) have also found extracts of *boiled egg* to be effective.

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additions. Failure has in most cases ultimately ensued (see Rats 1793, 1914, 1924, Chart II, and 2349, Chart III) and restoration been effected by the use of butter-fat or "butter oil." These failures have, however, only been noted after a far longer period than occurs where lard alone is used. Chart III shows the growth of rats supplied with beef-fat in the diet from a very early period. On this ration growth is continued for a far longer period than on the diets containing lard only. Growth has not, however, continued to complete maturity as it does with butter-fat in the food. For the present we interpret this as indicating that the content of the growth-promoting substance is so small that it ultimately becomes quantitatively inadequate.

Reports hitherto published include the following fats that have been tested from the standpoint considered in this paper:<sup>7</sup> with positive results—butter-fat,<sup>8,9</sup> egg yolk fat,<sup>8,9</sup> cod liver oil,<sup>10</sup> with negative results—lard,<sup>8,9</sup> olive oil,<sup>9</sup> almond oil.<sup>10</sup>

*Concentration of the growth-promoting substance.* The concentration of the effective substance contained in butter-fat and in beef-fat has been attempted by the following process:

A large quantity of absolute alcohol is saturated at 40° with the melted fat and then cooled to -15°. After standing in the freezing mixture for some time to allow the higher melting fats to crystallize, the solution is rapidly filtered on a large Buchner funnel. The clear alcoholic solution is then concentrated at 40° *in vacuo* until the alcohol is all removed. The clear, deep yellow oil which remains constitutes the "butter oil" (Fraction III) or "beef oil" used in these experiments.

The substance designated "Fraction I" was prepared by recrystallizing the part of the butter-fat filtered out as above described, until the glycerides liquid at room temperature were removed and a snow-white crystalline solid product obtained, which was equal to a little more than one-half of the butter-fat taken.

As a test procedure for the identification of "active" material we have employed the plan either of adding the substance to be

<sup>7</sup> An investigation of the behavior of the substance in butter-fat which exerts a growth-promoting influence is being conducted with reference to its diffusibility through rubber membranes, by Professor Gies at Columbia University.

<sup>8</sup> Osborne and Mendel: this *Journal*, xvi, p. 423, 1913.

<sup>9</sup> McCollum and Davis: *ibid.*, xv, p. 167, 1913.

<sup>10</sup> Osborne and Mendel: *ibid.*, xvii, p. 401, 1914.

tested to the diet of rats which have failed on the lard ration (see above) and watching for a resumption of growth, or of preventing the anticipated failure by an early addition of the investigated fraction to the ration.

Our experiments indicate clearly that the effective substance tends to concentrate in the fractions which we may term "butter oil" and "beef oil," respectively; *i.e.*, in those fractions which do not crystallize during the process described under the mode of preparation. The harder butter-fats (Fraction I) have failed to give evidence of growth-promoting properties (see Chart IV), in contrast with the liquid oil fraction (Fraction III) which promotes recoveries after failure on Fraction I (see Chart IV). That the growth-promoting substance of the beef-fat has been concentrated in the beef oil is shown in Chart V. Here again the purulent condition of the eyes, hitherto noted with the lard diets, also occurred with diets containing the inefficient fractions of the butter-fat and were cured by supplying the butter oil, as happened when the entire butter-fat was given. Whether these fat fractions deteriorate with age and exposure remains to be learned.

Preliminary to the determination of the quantities of "active" substance involved in the growth-promoting phenomena which have been recorded for the naturally occurring fats, we have made some investigation of the proportions of butter-fat, butter oil, cod liver oil, and beef oil needed to avert decline. Among hundreds of rats tested on the inadequate lard diets, one animal continued to thrive for the exceptional period of 252 days before the inevitable failure ensued; here too, however, addition of butter-fat induced a return of nutritive equilibrium. It is important to mention this unexpected endurance, unusual though it is, in evaluating the results observed with small additions of the effective fats. Our "standard" diets have as a rule contained 18 per cent of the latter. Many rats have been grown successfully on a diet containing 6 per cent of butter-fat; some on 3 per cent; a few on 1 per cent only. That these lower quantities approach the limits of adequacy is evidenced by the ultimate nutritive failure and the subsequent improvement following mere increase in the fat investigated. A few typical cases of such "quantitative" failures are recorded in Chart VI.

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The proportions of added "butter oil" and "beef oil" fractions selected were usually 6 per cent of the entire food, obviously representing a much larger addendum of the original fat. We have already shown that 6 per cent of cod liver oil also is satisfactory for growth.<sup>11</sup>

+ In comparing the numerous records of growth on diets containing butter-fat and beef-fat, respectively, we have gained the impression already referred to, that butter-fat is more effective in permitting growth than equivalent quantities of the beef-fat. Recoveries are less prompt and prolonged growth is less satisfactory when the latter is used. In this connection it may be observed that the yield of the liquid "oil" fraction from butter-fat is considerably larger than that from beef-fat. The findings in respect to the beef-fat explain the fact, which we have observed, that commercial olcomargarine also effects recovery in rats that have declined on the lard diets.

The new features of this communication may be summarized as follows:

The failure of lard to promote growth in the same manner as other natural fats (*i.e.*, butter-fat, egg yolk fat, cod liver oil) do, is not attributable to deteriorating changes arising from heat or chemical agents in the commercial manufacture of the product.

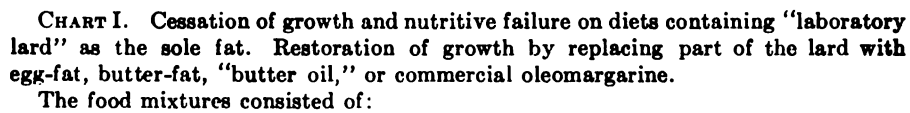
Heating butter-fat with steam does not destroy its growth-promoting efficiency.

Beef-fat ~~also~~ renders the inefficient diets used by us more suitable for producing growth in rats than does lard.

When butter-fat and beef-fat are subjected to fractional crystallization from alcohol, the growth-promoting factor remains in the mother liquor or "oil" fractions. The fractions containing the fats with high melting points are ineffective.

Some quantitative aspects of the growth-promoting efficiency of the natural fats are discussed.

<sup>11</sup> Osborne and Mendel: *ibid.*, xvii, p. 401, 1914, Chart III, Rats 1893 and 1898.



	per cent
Protein.....	18
Protein-free milk.....	28
Starch.....	24-29
Lard.....	7-28
Other fats.....	0-18



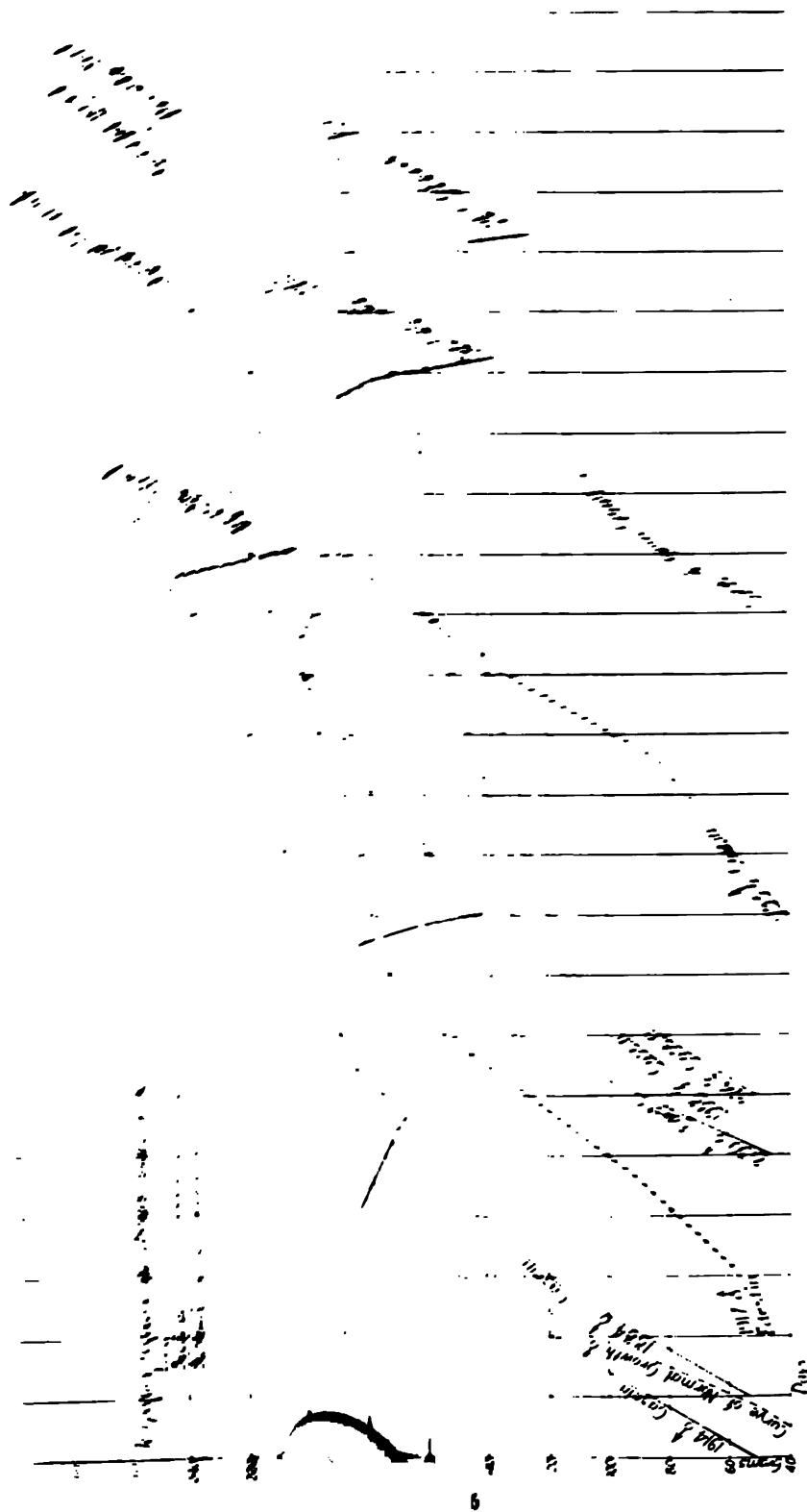


CHART II. Showing temporary recovery of growth when feed fat was added to feed after the feed failure. After the ultimate failure of feed fat, additions of butter products again restored growth. During the feed period the feed was replaced by equivalent quantities of butter products.

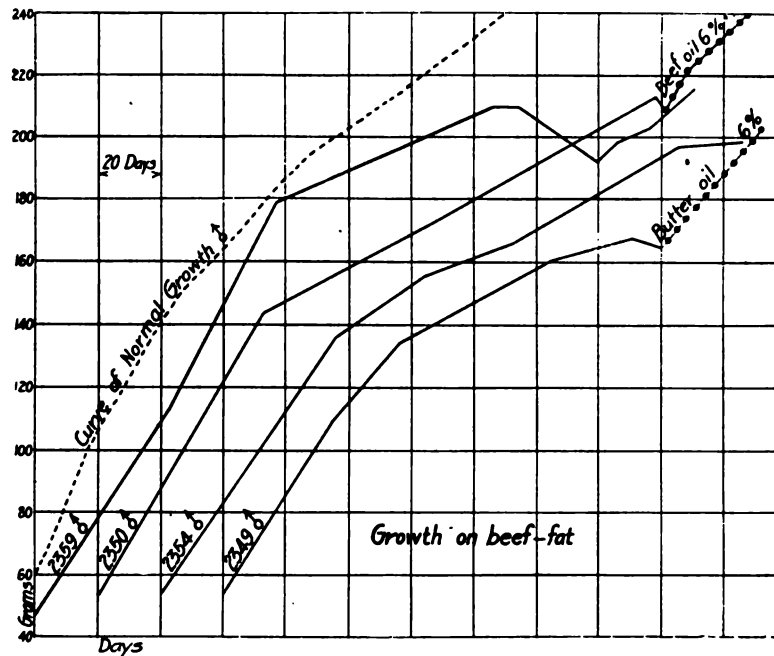


CHART III. Growth on diets containing beef-fat in the following mixture:

	per cent
Casein.....	18
Protein-free milk.....	28
Starch.....	29
Beef-fat.....	18
Lard.....	7

The effect on growth is far better than in the case of the lard diets; but it is inferior to that obtained with the butter-fat rations.



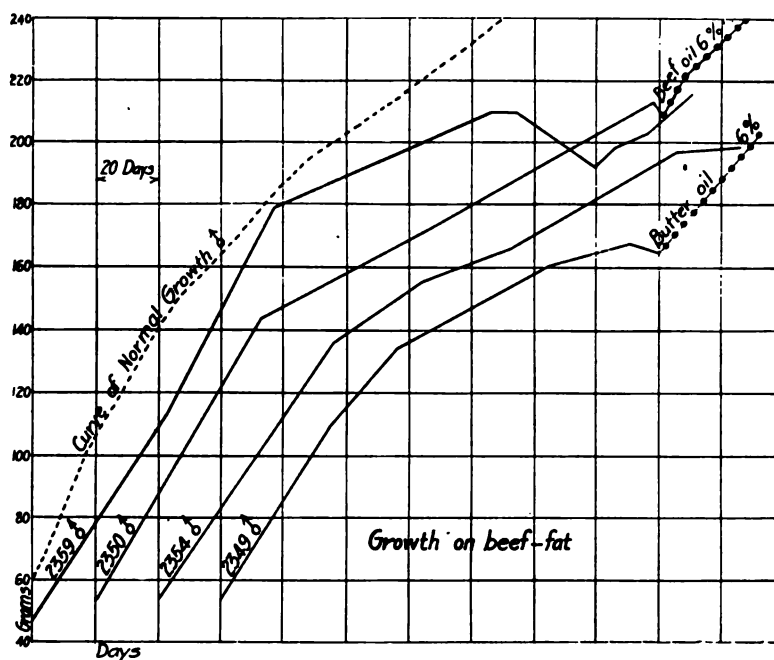


CHART III. Growth on diets containing beef-fat in the following mixture:

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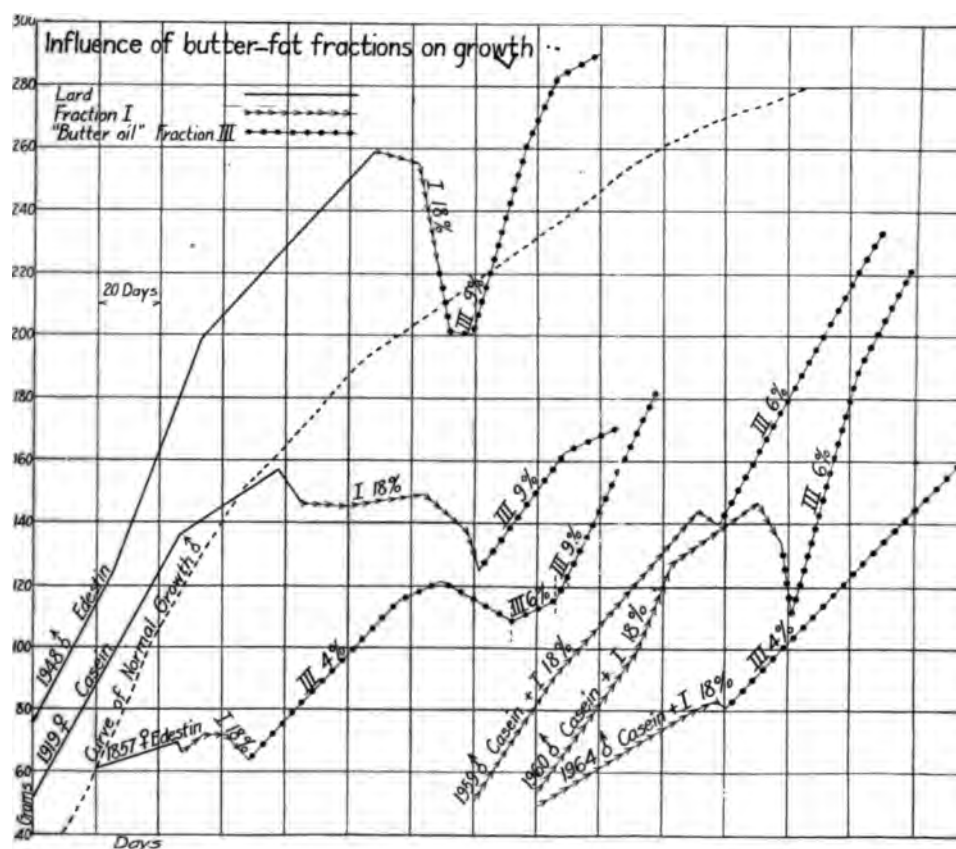


CHART IV. Showing cessation of growth on diets containing lard and the harder butter fat Fraction I, followed by recovery and renewal of growth when the "butter oil" Fraction III was fed.

The diets consisted of:

	per cent
Protein.....	18
Protein-free milk.....	28
Starch.....	20-29
Lard (with or without butter-fat fractions).....	9-28

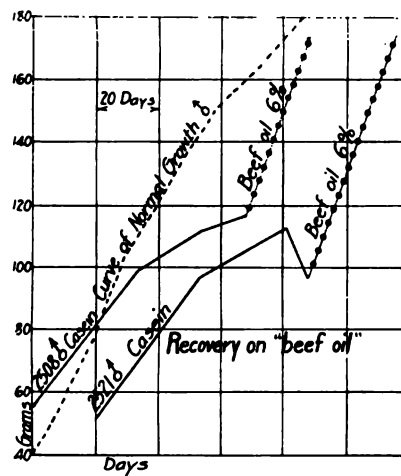


CHART V. Showing the growth-promoting properties of "beef oil" added to the food after cessation of growth on lard diets. The food mixtures resembled those described on other charts.

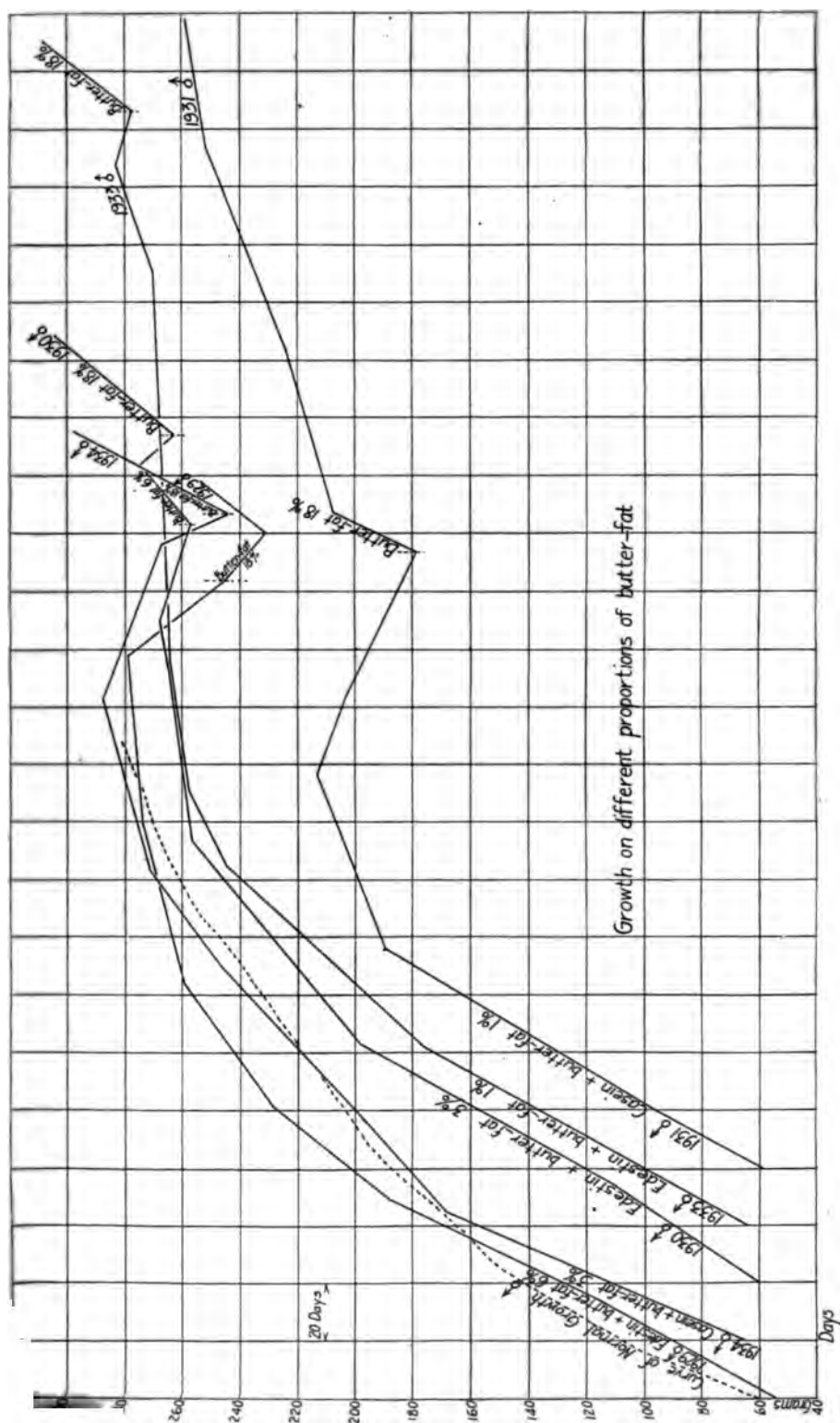


CHART VI. Showing the extent to which small proportions (1 per cent, 3 per cent, 6 per cent) of butter-fat are efficient in promoting growth and restoration and renewal of growth with larger proportions of butter-fat in the diet after decline had ensued.

**THE NON-PROTEIN NITROGENOUS COMPOUNDS OF  
THE BLOOD IN NEPHRITIS, WITH SPECIAL REF-  
ERENCE TO CREATININE AND URIC ACID.**

BY VICTOR C. MYERS AND MORRIS S. FINE.

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Medical School and Hospital, New York.)

(Received for publication, January 31, 1915.)

Since the introduction of simple methods for the determination of the total non-protein and urea nitrogen of the blood by Folin and Denis, a considerable literature has accumulated on the subject. Nephritis has been the pathological condition to receive special investigation, since it is in certain types of this disease that a retention occurs. Aside from the observations reported by Folin and Denis, however, practically no data have been presented in this connection dealing with the accumulation of creatinine and uric acid in the blood. Their data represent isolated observations on a number of cases. In the present paper consideration has been given especially to this phase of the question, but the study was made as intensive as conditions would permit on a few selected cases.

The non-protein and urea nitrogen of the blood has been found to fall within normal limits in many cases of nephritis. In those cases tending toward uremia, however, the values are increased and may reach figures of 350 mgm. per 100 cc. for the non-protein nitrogen, and 300 mgm. for the urea nitrogen.<sup>1</sup> The series of observations recently reported by Tileston and Comfort for both the non-protein and urea nitrogen and those by

<sup>1</sup> O. Folin and W. Denis: this *Journal*, xiv, p. 29, 1913; xvii, p. 487, 1914. C. B. Farr and J. H. Austin: *Jour. Exper. Med.*, xviii, p. 228, 1913. J. H. Agnew: *Arch. Int. Med.*, xiii, p. 485, 1914. F. C. McLean and L. Selling: this *Journal*, xix, p. 31, 1914. C. Frothingham, Jr., and W. G. Smillie: *Arch. Int. Med.*, xiv, p. 541, 1914. W. Tileston and C. W. Comfort, Jr.: *ibid.*, p. 620. C. B. Farr and E. B. Krumbhaar: *Jour. Am. Med. Assn.*, lxiii, p. 2214, 1914.



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Frothingham and Smillie for the non-protein nitrogen are especially illuminating on this subject.

As might be expected, cases of uremia are accompanied by an accumulation not only of urea, but also of uric acid and creatinine, as is well illustrated by the recent studies of Folin and Denis.<sup>2</sup> We<sup>3</sup> had already taken up this phase of the problem previous to the appearance of the paper by Folin and Denis; and at about the same time Neubauer,<sup>4</sup> in studying the impaired ability of the nephritic kidney to eliminate creatinine, reported a marked retention of creatinine in the blood in a case of uremia. A few observations on the creatinine content of dog's blood under different experimental conditions have been reported by Shaffer.<sup>5</sup>

### *Methods employed.*

The total solids were determined by collecting 0.3 to 0.6 of a gram of blood on a block of pressed filter paper and suspended by a wire hook from the stopper of a specially constructed weighing bottle. The Kjeldahl method was employed for the total nitrogen, while for the non-protein nitrogen, uric acid, creatinine, and creatine the technique was, with slight modifications, that described by Folin and Denis. In the urea estimations the concentrated urease<sup>6</sup> described by Van Slyke and Cullen was employed on either the fresh blood or residue of the same alcoholic filtrate • employed for the non-protein nitrogen. The ammonia thus obtained was aerated and subsequently determined colorimetrically as in the non-protein nitrogen estimation. The chlorides were determined by titration after removal of the protein (by coagulation in the presence of 1% acetic acid and subsequent treatment of the filtrate with a few drops of colloidal iron to remove the last trace of protein). A detailed description of

<sup>2</sup> Folin and Denis: *this Journal*, xvii, p. 487, 1914.

<sup>3</sup> V. C. Myers and M. S. Fine: *Proc. Soc. Exper. Biol. and Med.*, xi, p. 132, 1914.

<sup>4</sup> O. Neubauer: *München. med. Wchnschr.*, lxi, p. 857, 1914.

<sup>5</sup> P. A. Shaffer: *this Journal*, xviii, p. 535, 1914.

<sup>6</sup> D. D. Van Slyke and G. E. Cullen: *ibid.*, xix, p. 211, 1914. We are indebted to Dr. I. F. Harris of the Arlington Chemical Company for our supply of urease.

the technique we have employed in blood analysis may be found elsewhere.<sup>7</sup>

Twenty-five cases<sup>8</sup> are included in the present series, the first nine of which are of the retention type of nephritis. In two of these nine cases edema was present. The first three of these cases tabulated are of special interest since they are pronounced illustrations of different types of retention. The few cases other than nephritis are tabulated for comparison.

Case 1, suffering from mercuric chloride poisoning, showed a very pronounced retention of all the non-protein nitrogenous constituents, the figures for non-protein nitrogen, urea, uric acid, and creatinine being decidedly higher than in any case reported by previous workers. No urine was passed for the first five days and no appreciable amount for the first ten days. After decapsulation of the kidneys on the sixth day the renal activity appeared to improve, and at one time it had sufficiently recovered to cause a reduction in the concentration of the creatinine from 33.3 to 14.8 mgm. per 100 cc.; but with the decline of the patient, the kidneys became less active and the creatinine again increased. The quite favorable output of total nitrogen was insufficient at any time, however, to reduce materially the non-protein and urea nitrogen of the blood, despite the favorable influence on the uric acid and creatinine. Although the highest concentration of uric acid and creatinine, as well as very high figures for non-protein and urea nitrogen, were found on November 20, uremic symptoms were not observed until a week later. This would seem to lend support to the current view that uremia is not a result wholly of the retention of these nitrogenous waste products.<sup>9</sup>

Case 2 is interesting as illustrative of the condition of the blood and urine in a very severe case of interstitial nephritis with uremia but no edema. In this case there was a gradual decrease in the

<sup>7</sup> Papers in *The Post-Graduate*, 1914-15, and collected as *The Chemical Composition of the Blood in Health and Disease*.

<sup>8</sup> Practically all the cases here reported were patients in the medical wards of this Hospital. We are indebted to the Director of the Department, Dr. Quintard, to Drs. Chace, Kast, and Halsey for many courtesies extended in connection with these studies; to the resident physician, Dr. W. G. Lough, and to Dr. F. D. Gorham for aid in following the cases and arranging the case histories.

<sup>9</sup> See F. W. Peabody: *Arch. Int. Med.*, xiv, p. 236, 1914.

Male, 25 years of age, patient of Drs. Stieglitz, Squier, and Chace.  
Blood pressure: systolic, 155; diastolic, 70.  
Characteristic urinary findings: approximately 0.16 per cent albumin, few hyaline and granular casts. Symptoms of uremia developed on Nov. 28.  
On Dec. 2, an infusion of one liter of physiological saline was given.

Case 2. (I. D.) Chronic interstitial nephritis, uremia.

BLOOD ANALYSES										URINARY ANALYSES DAILY AVERAGES						
DATE (1914-15)	Total solids	Total N	Non-protein N	Urea N	Uric acid	Creatinine	Creatinine	Chlorides as NaCl	DATE (1914-15)	Per 100 cc. of blood						
										gm.	gm.	mgm.	mgm.	mgm.	mgm.	gm.
Dec. 10.	17.6	2.98	181	139	6.3	10.0	7.8		Dec. 13-14	1050			0.07	0.39		0.83
Dec. 20.	Spinal fluid		180	143	0	4.8	1.0		Dec. 18-20	1160	1.012	5.57	0.14	0.37	0.10	0.72
Dec. 21.	16.4	2.46	199	134	12.5	14.5	11.5	0.48	Dec. 20-23	687	1.013	3.65	0.07	0.29	0.07	0.63
Dec. 26.	14.7	2.18	244	151	15.4	17.7	20.8	0.48	Dec. 23-26	660	1.012	3.64	0.16	0.17	0.16	0.64
Dec. 30.	14.6	2.21	267	170	21.0	16.1	25.5	0.46	Dec. 26-30	550	1.012	2.91	0.13	0.13	0.19	0.53
Jan. 4....	13.7	1.92	297	208	27.0	20.0	31.7	0.50	Dec. 30 to Jan. 3....	359	1.009	1.75	0.09	0.15	0.16	0.99
									Jan. 3-4....	140	1.012	1.40		0.07	0.05	0.14

Small, fairly well nourished female, 17 years of age; moderate dilatation of heart, no edema.

Average blood pressure: systolic, 170; diastolic, 130.

Phenolphthalein output: 0 (3 determinations).

Characteristic urinary findings: 0.2 to 0.3 per cent albumin, few hyaline and granular casts, pus cells and mucus.

Ate practically no food during period of observation. 1800 to 2000 cc. of saline and glucose given per rectum daily; considerable vomiting.

Died, Jan. 4, 1915.

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activity of the kidney with a corresponding retention in the blood. As will be noted, the figure for the uric acid just previous to death, 27 mgm., is very high, even exceeding those reported for Case 1.

Case 3 was found at autopsy to be typically of the interstitial type, although, as distinguished from Case 2, there was a marked accumulation of fluid during the last two weeks of life which enabled us to secure large samples of pleural, ascitic, and subcutaneous fluids for analysis. Uremic symptoms did not show, however, until just preceding death, and it should further be noted that there was not a very marked accumulation of waste products until shortly before the fatal termination. Although the urea and uric acid reached a fairly high level, the figure of 5.3 for the creatinine is not high when compared with the other cases.

Marshall and Davis<sup>10</sup> have pointed out that urea is quite uniformly distributed throughout the body. This is certainly true here with regard to the body fluids, as shown in the tabulated data on this case. A similar state of equable distribution throughout the body fluids and tissues was observed for creatinine. The same may be said in general for uric acid, if we except the spinal fluid, the difference here recorded being of interest in view of the position of relative isolation held by this fluid.<sup>11</sup>

#### DISCUSSION.

The points of special interest in the tabulated data below bear brief discussion. Very high concentrations of uric acid and creatinine may be encountered in nephritis with retention of nitrogen. Uric acid has been found as high as 27.0 mgm., and creatinine as high as 33.3 mgm. per 100 cc. of blood.

That the retention of the well known end-products of protein metabolism are not in themselves the cause of uremic symptoms would appear evident from the case of mercury bichloride poisoning, in which uremic symptoms did not appear until more than a week after maximum concentrations of these substances in general had been attained. The possibility that a decomposition product of creatinine, such as methylguanidine, might play a part

<sup>10</sup> E. K. Marshall, Jr., and D. M. Davis: this *Journal*, xviii, p. 53, 1914.

<sup>11</sup> See papers by H. Cushing, L. H. Weed, and P. Wegefarth: *Jour. Med. Research*, xxxi, pp. 1-176, 1914.

Case 3. (E. E.) Chronic interstitial nephritis, uremia.

DATE (1914-15)	Fluid or tissue	BODY FLUID OR TISSUE ANALYSES							URINARY ANALYSES	
		Total solids	Total N	Chlorides as NaCl	Non-protein N	Urea N	Uric acid	Creatinine	Creatinine	DATE (1914-15)
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	Averages
Dec. 18	Blood	17.8	2.43	0.71	61	33	1.0	4.2	8.0	Dec. 23-28..... Dec. 29-Jan. 6... Dec. 29-Jan. 11..
Dec. 29		15.9	2.43	0.71	59	33	4.2	4.1	7.7	
Jan. 9		16.8	2.50	0.63	81	59	8.0	4.6	7.0	
Jan. 19		13.7	1.98	0.73	140	100	15.4	5.3	21.3	
Jan. 12	Ascitic fluid..... Pleural fluid..... Subcutaneous fluid.....	3.7	0.45	0.85	81	74	11.3	4.2	2.7	
		3.4	0.48	0.84	81	74	10.5	4.0	3.5	
		1.6	0.20	0.85	81	74	10.7	4.7	1.9	
		0.45	0.79			100	18.0	6.0	15.3	
Jan. 19	Pleural fluid..... Subcutaneous fluid..... Spinal fluid..... Pectoral muscle..... Liver..... Heart muscle..... Spleen.....	0.44	0.84			100	16.7	6.3	11.3	
			0.99			100	18.0	6.0	14.2	
						100	4.4	4.4	4.4	
						125	8.0	6.8	324.0	
				0.42		116	10.0	5.3	50.5	
				0.36		100	18.0	6.8	148.0	
				0.43		115	12.6	7.8	26.7	

Small, poorly nourished female, 30 years of age; weight, Nov. 2, 78 lbs.; marked dilatation of heart.  
 Average blood pressure: systolic, 225; diastolic, 160.  
 Phenolsulphonphthalein output: 10 per cent, Nov. 12.  
 Characteristic urinary findings: 0.2 to 0.3 per cent albumin, many hyaline and granular casts, pus cells.  
 Ate very little food, only lemonade from Jan. 11.  
 Wassermann + + +.  
 Edema began Dec. 6. Dyspnea developed five days before death.  
 Died, Jan. 19, 1915. Examination of tissues bore out the diagnosis.  
 Average daily NaCl output during last week of life: 0.4 gm.

CASE	AGE	SEX	CLINICAL DIAGNOSIS	DATE (1914-15)	BLOOD ANALYSES										REMARKS
					Per 100 cc.										
					Total solids	Total N	Chlorides as NaCl	Non-protein N	Urea N	Uric acid	Creatinine	Creatinine			
4. W.O.C.	33	M	{ Chronic interstitial nephritis, uremia }	Mar. 7			0.54	292	200	10.5	9.0		Well nourished, cardiac dilatation, mitral regurgitation. Average systolic pressure: 200. Saline infusion preceding 2d Cl test. Phthalein output: less than 1 per cent. Edema of lower extremities. Urine: moderate amount of albumin, few hyaline casts.		
				Mar. 9	12.1	2.60	0.67	207	182	11.4	16.6	7.0			
5. M.K.	42	M	{ Chronic interstitial and parenchymatous nephritis, uremia }	May 4									Died, Mar. 10, 1914. Well nourished, anemic; weight 132 lbs. Mitral insufficiency. No edema. Urine: moderate amount of albumin, moderate number of granular casts. In coma, May 13; decapulation of both kidneys. Died on operating table.		
				May 7	12.0	1.64	0.64	155	120	8.0	10.0	7.0			
				May 13 a.m.	13.8	1.79	0.51	184	140	13.7	13.9	3.8			
				May 13 p.m.			0.61	226	170	14.0	14.7	15.2			
6. J.S.	50	M	{ Nephritis of mixed type, uremia }	Nov. 23	20.8	3.11		169	81	13.4	7.4	13.1	Blood pressure: systolic 112; diastolic 88. Died in uremic coma. On Jan. 6, patient was dyspneic and uræmic. Phthalein output: 0. Systolic pressure, 250; diastolic, 160. By Jan. 20 there was considerable improvement. Phthalein output: 31 per cent. Systolic pressure, 200; diastolic, 130. Urine: moderate amount of albumin, occasional hyaline and granular casts.		
7. L.P.	57	M	{ Interstitial nephritis, uremia }	Jan. 6	16.5	2.46		129	80	8.0	4.8	8.9	Moderate cardiac hypertrophy. Systolic pressure, 230; diastolic, 130. Phthalein output: between 3 and 4 per cent. Urine: moderate amount of albumin, moderate number of hyaline and granular casts.		
				Jan. 7	Spinal fluid					1.5	3.9	0.5			
				Jan. 23	18.0	2.56	0.75	65	34	2.2	3.6	7.9			
8. T.D.	34	M	{ Interstitial and vascular nephritis, uremia }	Feb. 18	17.0	2.56		59	36	3.6	3.1	6.7	On Jan. 6, patient was dyspneic and uræmic. Phthalein output: 0. Systolic pressure, 250; diastolic, 160. By Jan. 20 there was considerable improvement. Phthalein output: 31 per cent. Systolic pressure, 200; diastolic, 130. Urine: moderate amount of albumin, occasional hyaline and granular casts.		
				Jan. 22	17.4	2.65		93	60	7.0	7.8	4.2			
				Jan. 27	16.9	2.62		113	67	7.0	10.0	10.3			
				Jan. 30	15.3	2.37	0.67	124	77	6.8	8.0	5.2			
				Feb. 6	14.2	2.14	0.62	99	72	6.0	8.4	4.1			
				Mar. 9	15.0	2.24		97	9.1	11.0	5.6				

9, W.G.	8	M	Acute nephritis of mixed type with uremia and edema	May 14 July 8 July 9 July 10 July 13 July 24 July 30	14.5   2.21 14.4   1.99 13.4   1.73 12.1   1.35 13.0   1.70 13.1   1.64	06 100 119 123 127 131	32 71 83 86 94 109	2.5 3.9 4.7 0.0 5.5 6.0	1.4 2.9 5.4 7.0 6.0	8.9 5.0 6.0 7.0 3.7 5.1	Extensive edema. Phthalein output: 3 to 8 per cent. Urine: large amount of albumin, many hyaline and granular casts, few pus cells and erythrocytes.
10, I.R.	45	M	Interstitial and vascular nephritis	Dec. 28 Jan. 11	19.2   2.94 17.7   2.94	42 59	5.5 6.0	4.6 4.2	3.6 4.1		Edema of ankles. Phthalein output: 47 per cent. Urine: moderate amount of albumin, moderate number of casts. 10 gm. NaCl 3 hrs. preceding 2d test. Phthalein output 34 per cent. Urine: trace of albumin, occasional hyaline casts. Blood transfusion, Mar. 27. 10 gm. NaCl on Apr. 1, 14 hrs. preceding test.
11, W.McL.	42	M	Chronic interstitial nephritis, edema	Mar. 31 Apr. 1 May 5 May 12 May 14 May 23 June 3 June 8 June 9 June 10	15.9 19.2   2.57 17.9   2.50 17.6   2.57 17.5   2.47 18.1   2.46 17.5	0.72 0.75 0.72 0.61 0.59 0.62	20 29 27 38 3.0 2.0 2.8	4.0 4.5 4.5 4.6 3.4 3.0 2.0	2.1 1.8 1.7 1.3 1.6 2.0	7.7 9.1 9.0 9.7	Extensive edema. Blood pressure varied from 190 (diastolic, 130) to 150 (diastolic, 100). Phthalein output: 33 per cent. Urine: moderate amount of albumin, many hyaline and granular casts. Died. Marked edema of extremities. Phthalein output: 3 per cent. Urine: moderate amount of albumin, many hyaline and granular casts, few pus cells. Died.
13, H.S.	37	F	Chronic glomerular nephritis	Mar. 13	13.9   1.73 17.3   2.36	48 39	2.9 4.1	1.8 3.3	1.8 6.7		Slight edema of feet and ankles. Average blood pressure: systolic, 180; diastolic, 95. Phthalein output 16 per cent. Urine: few hyaline casts, trace of albumin. No edema.
14, L.R.	52	F	Chronic interstitial nephritis	Nov. 23	17.3   2.36	39	17	4.1	3.3	6.7	Average blood pressure: systolic, 110; diastolic, 70. Phthalein output: 10 to 22 per cent. Urine: trace of albumin, occasional hyaline and granular casts.
15, F.S.	67	M	Chronic parenchymatous nephritis	Dec. 15	21.4   2.98	34	10	4.2	1.5	9.0	Slight edema of feet and ankles. Systolic pressure, 240; diastolic, 100. Phthalein output: 56 per cent. Urine: trace of albumin, occasional hyaline casts.
16, L.B.	64	F	Chronic interstitial nephritis	Dec. 28	18.8   2.94	33	2.2	3.1	5.3		Systolic pressure, 240; diastolic, 100. Phthalein output: 56 per cent. Urine: trace of albumin, occasional hyaline casts.



CASE	AGE	SEX	CLINICAL DIAGNOSIS	DATE (1914-15)	BLOOD ANALYSES							REMARKS	
					Total solids	Total N	Chlorides as NaCl	Non-protein N	Urea N	Uric acid	Creatinine		
					gm.	gm.	gm.	gm.	gm.	gm.	gm.		
Per 100 cc.													
17, L.S.	55	M	{ Chronic inter- stitial ne- phritis, ac- cording to aortic insuffi- ciency	Jan. 6	17.5	2.43	30		6.3	2.1	7.7	{ Systolic pressure, 170; diastolic, 80. Urine: trace of albumin, occasional hyaline and granular casts.	
18, H.K.	26	M		{ Acute paren- chymatous and tubular nephritis	Sept. 10	19.7	3.04	33	15	2.6	0.9	10.3	{ Edema of both ankles. Systolic pressure, 140; diastolic, 80. Phthalein output: 33 per cent. Urine: large amount of albumin, many hyaline and granular casts.
19, A.K.	52	F	{ Chronic inter- stitial neph- ritis	Sept. 17	20.7	3.24	67	28	3.6	0.9	13.9	{ Systolic pressure, 132; diastolic, 106. Phthalein output: 39 per cent. Urine: trace of albumin, occasional granular casts.	
20, I.W.	16	M	Pyelitis	Jan. 6	20.6	2.88	27		0.5	2.9	5.9	{ Systolic pressure, 130; diastolic, 73. Urine: trace of albumin, many pus cells.	
21, J.B.	22	M	Syphilis	Dec. 8	21.1	3.10	30	14	2.0	1.6	7.8		
22, B.		M	Gangrene	May 22	20.1	0.55	27	12	1.6	1.4	8.6		
				June 3	17.1	2.82	0.64	46	30	2.9	1.8	9.0	
23, G.A.	43	M	Arthritis	June 25					1.0	1.0	9.9		
				June 27					2.2	0.9	7.0		
24, F.	25	F	Diabetes	May 22	14.8	2.11	0.51	22	13		1.5	7.9	{ May 22. Blood sugar: 0.26 per cent. May 25. Blood sugar: 0.37 per cent.
				May 25	15.6	2.18	0.51	37	21	0.7	1.5	10.2	{ Uric acid in muscle tissue obtained at autopsy: 0.7 mgm. per 100 cc. of blood.
25, T.B.	57	M	Gout	Dec. 23	20.7	3.20	0.64	36	12	4.2	1.4	9.3	{ Phthalein output: 35 per cent.
				Dec. 30				42	12	4.2	1.9	9.4	

in the development of uremic symptoms needs to be considered, although there are as yet no definite data to support this view.<sup>12</sup>

In six cases of gout examined in this laboratory, uric acid in 100 cc. of blood ranged from 3.8 to 5.8 mgm. The far higher values for uric acid in the above cases of uremia, in which gouty symptoms were absent, are of interest in view of the controversy existing with regard to the relation between the retention of uric acid in gout and the clinical symptoms.<sup>13</sup>

TABLE I.  
*Summary of data on uric acid, creatinine, and creatine in the blood.*

CASE	CLINICAL DIAGNOSIS	URIC ACID	CREATININE	CREATINE
		Per 100 cc.		
		mgm.	mgm.	mgm.
1	Hg poisoning, uremia.....	15.0	33.3	19.4
2	Chronic interstitial nephritis, uremia.....	27.0	20.0	31.4
3	Chronic interstitial nephritis, uremia, edema....	15.4	5.3	21.3
4	Chronic interstitial nephritis, uremia.....	11.4	16.6	
5	Chronic interstitial and parenchymatous nephritis, uremia.....	14.0	14.7	15.2
6	Nephritis of mixed type, uremia.....	13.4	7.4	13.1
7	Interstitial nephritis, uremia.....	8.0	4.8	
8	Interstitial and vascular nephritis, uremia.....	9.1	11.0	
9	Acute nephritis of mixed type, uremia, edema....	5.5	7.0	
10	Interstitial and vascular nephritis.....	5.5	4.6	
11	Chronic interstitial nephritis, edema.....	4.0	2.1	
12	Chronic vascular and tubular nephritis.....	4.5	1.8	
14	Chronic interstitial nephritis.....	4.1	3.3	
15	Chronic parenchymatous nephritis.....	4.2	1.5	
17	Chronic interstitial nephritis, aortic insufficiency	6.3	2.1	

Another point which appears to possess some little significance is the increase in the creatine content of the blood in those cases showing a very marked rise in the uric acid. There was not a

<sup>12</sup> In this connection the papers by N. E. Ditman and W. H. Welker: *New York Med. Jour.*, lxxxix, pp. 1000, 1046, 1091, 1134, 1909, are worthy of note. Also W. M. Kraus: *Arch. Int. Med.*, xi, p. 613, 1913.

<sup>13</sup> See M. S. Fine and A. F. Chace: *Jour. Pharm. and Exper. Therap.*, vi, p. 219, 1914; *Jour. Am. Med. Assn.*, lxiii, p. 945, 1914.

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complete retention of uric acid, but even if no uric acid were eliminated during the last days of life, the amount of retention, if anything, exceeded the probable endogenous formation (Cases 2 and 3). From the data presented on Cases 3 and 25, we may assume that uric acid is quite evenly distributed throughout the fluids and tissues. Since the increased creatine is suggestive of increased tissue destruction,<sup>14</sup> it is possible that this in part explains the very high uric acid.

The distribution of these products in the spinal fluid is worthy of note; whereas urea and creatinine approach the concentrations found in the other fluids in uremia, the creatine and uric acid are very low or practically absent.

The comparison between the retention figures for uric acid and creatinine is of interest. In some cases the creatinine is decidedly higher than the uric acid, while in others the reverse is true (Table I). In the majority of the cases a high creatinine is found to accompany a very high urea, while in those cases in which the creatinine is low the urea is generally low. There appears to exist some parallelism, then, between the accumulation of urea and creatinine, and, as pointed out above, also between the uric acid and creatine, although it has not been possible to correlate these parallelisms with any specific types of nephritis.

<sup>14</sup> Myers and Fine: this *Journal*, xv, p. 283, 1913.

# STUDIES ON A METHOD FOR THE QUANTITATIVE ESTIMATION OF CERTAIN GROUPS IN PHOSPHOLIPINS.

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In 1903 Baskoff<sup>1</sup> published a method for the quantitative estimation of glycerol and the nitrogen complex. He dissolved lecithin in absolute alcohol and saturated this with hydrochloric acid gas. The solution was heated with a reflux condenser until hydrolysis and esterification of the fatty acids had taken place. The alcohol was then almost entirely removed by heating, the solution diluted with water, and the esters of the fatty acids were shaken out with ether. The solution at this stage contained the nitrogen complex, glycerophosphoric acid, free phosphoric acid, and glycerol. A portion of this was treated according to the method of Stanek-Kiesel<sup>2</sup> with potassium triiodide, and the nitrogen of the precipitate was determined by Kjeldahl. It gave 1.09 per cent, which represents 11.07 per cent choline. A second portion was evaporated to dryness and extracted with absolute alcohol to remove the choline hydrochloride. The residue was treated with water and again taken down to dryness, a process repeated several times. It was finally transferred to a Fanto-Zeisel<sup>3</sup> apparatus where it was concentrated to 5 cc., 15 cc. of hydriodic acid were added, and it was heated to boiling. At the same time a stream of CO<sub>2</sub> was passed through the whole apparatus. The vapor of isopropyl iodide which escaped from the upright cooler was led for purification from hydriodic acid vapor through a wash bottle containing red phosphorus under water, and finally

<sup>1</sup> A. Baskoff: *Ztschr. f. physiol. Chem.*, lvii, p. 435, 1908.

<sup>2</sup> V. Stanek: *ibid.*, xlvi, p. 283, 1905.

<sup>3</sup> S. Zeisel and R. Fanto: *Ztschr. f. anal. Chem.*, xlii, p. 549, 1903.

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into an alcoholic solution of silver nitrate. The silver iodide was weighed, and by calculation gave the amount of glycerol. Baskoff found in lecithin 10.61 per cent, calculated as  $C_3H_5(OH)_3$ ; the theoretical amount is 11.8 per cent as  $C_3H_5(OH)_3$ , 5.2 per cent as  $C_3H_5$ , on Trier's<sup>4</sup> formula.

The above work suggested the possibility of splitting off the glycerol group and the methyl groups attached to the nitrogen in one operation, but at different temperatures, by means of hydriodic acid. Preparations of phospholipins, *i.e.*, lecithin, the alcohol-soluble compound, and kephalin, the alcohol-insoluble compound, were made according to Waldemar Koch's<sup>5</sup> method, except that care was taken to use only anhydrous solvents. By this method, after repeated purification, the alcohol-soluble compound gave by Arnold-Gunning Kjeldahl for N = 1.91 per cent, and by the Neumann method for P = 3.05 per cent weighed as  $Mg_3P_2O_7$ ; the alcohol-insoluble preparation gave N = 1.89 per cent; P = 3.73 per cent.

The following table shows the results obtained by various investigators of these substances:

	N per cent	P per cent
<b>KEPHALIN</b>		
Thudichum.....	1.68	4.27
W. Koch <sup>5</sup> .....	1.78	3.84
Neubauer <sup>6</sup> .....	1.65	3.45
My preparation.....	1.91	3.05
<b>LECITHIN</b>		
Diakonow <sup>7</sup> .....	1.8	3.8
Thierfelder <sup>8</sup> .....	2.08	3.97
McLean <sup>9</sup> .....	1.88	3.95
Eppler.....	2.09	3.95
Thudichum.....	2.03	4.29
W. Koch.....	1.80	3.79
Erlandsen <sup>10</sup> .....	1.87	3.95
My preparation.....	1.89	3.73

<sup>4</sup> G. Trier: *Ueber einfache Pflanzenbasen und ihre Beziehungen zum Aufbau der Eiweissstoffe und Lecithine*, Berlin, 1912.

<sup>5</sup> W. Koch: *Am. Jour. Physiol.*, xi, p. 319, 1904.

<sup>6</sup> E. Neubauer: *Biochem. Ztschr.*, xxi, p. 321, 1909.

<sup>7</sup> Diakonow: *Med. chem. Untersuch.*, 1867, p. 21.

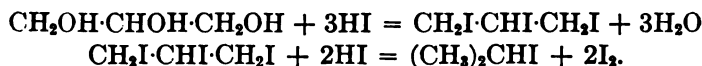
<sup>8</sup> H. Thierfelder: *Ztschr. f. physiol. Chem.*, xlvi, p. 518, 1905.

<sup>9</sup> H. McLean: *ibid.*, lvii, p. 297, 1908.

<sup>10</sup> A. Erlandsen: *ibid.*, li, p. 71, 1907.

While these figures vary considerably among themselves, the ratio 1 : 1 between N and P is fairly well maintained and shows that these phospholipins, although obtained by sundry methods of extraction, nevertheless belong to the class of mono-amino-mono-phosphatides, as stated by Erlandsen.

Before applying the method for determining the glycerol in these preparations, controls were run with pure glycerol. The glycerol (Merck's preparation) was so diluted that 0.5 cc. contained 0.2 to 0.3 gram of glycerol. This was placed in a small round-bottomed flask with 1 gram of ammonium iodide and 15 cc. of hydriodic acid, and connected with an upright 15 inch condenser having a thermometer and a side tube, leading to a Geissler bulb which contained 2 per cent solutions of sodium carbonate and potassium arsenite, as recommended by Klinger and Kreutz.<sup>11</sup> The flask containing the glycerol was heated in a glycerine bath to 112° to 113°, at which temperature a cloud appeared in the silver nitrate solution. The heating was continued at this temperature until the precipitate became crystalline and settled, leaving the supernatant liquid perfectly clear. The reaction probably takes place according to the equations:



AMOUNT OF GLYCEROL USED	AMOUNT OF AgI OBTAINED	AMOUNT OF GLYCEROL FOUND	PER CENT
<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
0.2466	0.6323	0.2479	100.4
0.2292	0.5296	0.2077	90.62
0.2306	0.5167	0.2026	87.8
0.0719	0.1800	0.0704	98.4

The time required for completion of the reaction was two to three hours, the temperature remaining constant at 112° to 113°. The temperature was finally allowed to rise to 120°, at which point if no further precipitation took place, the process was discontinued. The weight of silver iodide multiplied by the factor

<sup>11</sup> H. Klinger and A. Kreutz: *Ann. d. Chem.*, ccxlix, p. 147, 1888.

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0.3922 gives the weight of glycerol found. This method was a combination of the Fanto-Zeisel and Hewitt and Moore<sup>12</sup> methods.

A blank gave negative results and warranted the conclusion that the Geissler bulb was holding back all hydriodic acid and iodine.

The phospholipins were then tested in the apparatus. Keph-  
alin was tested first and gave a precipitate at 112°, corresponding  
in every detail to that obtained with glycerol. Even on heating  
a second time no turbidity appeared in the silver nitrate solution.

KEPHALIN NO. USED	WT. OF AgI. gm.	GLYCEROL CALCULATED gm.	GLYCEROL per cent
1. 0.2181	0.0507	= 0.0199	= 9.11
2. 0.7260	0.1517	= 0.0595	= 8.21
3. 0.3242	0.0628	= 0.0246	= 7.62

On raising the temperature no further precipitation in a fresh  
silver nitrate solution took place. This indicates that no methyl  
group was liberated.

No.	gm.	gm.	gm.	per cent
1.	0.2438 lecithin	0.0563	AgI at 112° = 0.0220	glycerol = 9.05
1 a.	0.2438 lecithin	0.0368	AgI at 180° = 0.00235	methyl = 0.96
2.	0.2235 lecithin	0.0548	AgI at 112° = 0.0215	glycerol = 9.61
3.	0.2615 lecithin	0.0668	AgI at 112° = 0.0262	glycerol = 10.01
4.	0.4972 hydrolyzed lecithin	0.1214	AgI at 112° = 0.0476	glycerol = 9.57
5.	0.4972 hydrolyzed lecithin	0.1135	AgI at 112° = 0.0445	glycerol = 8.95
6.	0.4972 hydrolyzed lecithin	0.1182	AgI at 112° = 0.0464	glycerol = 9.32

Average for Nos. 1, 2, and 3, unhydrolyzed = 9.55 per cent;  
for 4, 5, and 6, hydrolyzed according to Baskoff's method = 9.38  
per cent. It is evident that this process is unnecessary, the hy-  
driodic acid alone being sufficient.

In every instance after apparently complete precipitation of  
iodide from the glycerol had taken place, the temperature was  
run up slowly to 300°, but no further clouding of the fresh silver  
nitrate solution took place except in 1 a, where a precipitate was  
obtained. This was, however, too much to be wholly glycerol  
and too little for three methyl groups. Two facts had become  
evident: the glycerol group in the phospholipins under consider-

<sup>12</sup> J. T. Hewitt and T. S. Moore: *Jour. Chem. Soc.*, lxxxi, p. 320, 1902.

ation is very easily and almost quantitatively split off by hydriodic acid; and, second, either the method is ineffective where imide methyl groups are concerned; or there is no methyl in the substance. The first fact will necessitate the recalculation of Waldemar Koch's results. In the case of kephalin:

0.3488 gm. substance; 0.0945 gm. AgI = 0.0378 gm. glycerol = 10.8 per cent.

instead of

0.3488 gm. substance; 0.0945 gm. AgI = 0.006036 gm. CH<sub>3</sub> = 1.73 per cent.

This admits of no methyl group in kephalin and agrees with Winterstein's<sup>13</sup> finding of 10.2 per cent glycerol, or 1.63 per cent CH<sub>3</sub>, as given by this method. In the case of egg lecithin Koch got a precipitate of 0.0760 gram of silver iodide from 0.320 gram of the substance below 240°. Calculating this as glycerol, the yield is 9.5 per cent, and leaves for methyl only 4.3 per cent instead of 5.80 per cent, or two methyl groups instead of three.

These irregularities and inconsistencies suggested the necessity of testing choline, which has so often been called the alkaloidal base of lecithin. Choline chloride was prepared by the method of Wurtz,<sup>14</sup> as modified by Renshaw.<sup>15</sup>

Attempts were made to determine the methyl groups in this preparation by the use of the Fanto-Zeisel method given above, but no adequate cleavage of the methyl group took place. According to Decker,<sup>16</sup> dry heating is necessary and this is the chief advantage of the double barrel distilling flask used in the Herzig and Meyer method. By using separate cups filled with paraffin the temperature can be absolutely controlled, as is not the case in the sand bath, where the temperature about the immersed flask was found to vary 40° to 50° in spots a few inches apart. The tube connecting the two flasks was bent into an inverted U, so that the flasks were immersed nearly to the stoppers in paraffin. The temperature was allowed to rise slowly to 140°, where it was held for half an hour, then allowed to rise again to 180° to

<sup>13</sup> E. Winterstein and O. Hiestand: *Ztschr. f. physiol. Chem.*, liv, p. 300, 1907-08.

<sup>14</sup> A. Wurtz: *Ann. d. Chem.*, Supplement, vi, p. 200, 1868.

<sup>15</sup> R. R. Renshaw: *Jour. Am. Chem. Soc.*, xxxii, p. 128, 1910.

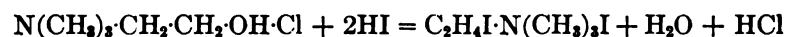
<sup>16</sup> H. Decker: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 2895, 1903.



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185°, at which point a precipitate was usually formed in the silver nitrate solution. Here the temperature was held until the reaction was over, a process lasting four to five hours. The precipitate was then treated as with glycerol.

The reaction takes place in two stages. According to Wurtz, the change of the alkyl groups of choline into the iodide form occurs between 120° to 150°. The boiling point of hydriodic acid is 127°, slightly raised in this instance by the presence of the ammonium iodide.



This reaction is slow and requires time. It seems probable that it is because this reaction has been incomplete that repeated successive heatings are necessary. No way of knowing when this reaction is complete has been discovered, however. At a higher temperature dissociation takes place, and methyl iodide, which decomposes less readily than ethyl iodide, is split off, and passes over into the alcoholic silver nitrate, where it is precipitated.

Besides the substitution of a paraffin bath for the sand bath, some other modifications were introduced: a current of water heated to 40° to 50° ran through the condenser while the Geissler bulb containing 2 per cent solutions of sodium carbonate and potassium arsenite was immersed in water heated to 50° to 60°; a second flask containing the arsenite solution, also heated, was introduced as a precautionary measure; and finally the gases were bubbled through a tall cylinder of alcoholic silver nitrate by means of a modified Folin tube. This effected a more complete precipitation. A second, third, and even fourth heating increased the yield, although the evidence of the settling of the precipitate in each previous heating had been taken as sufficient proof that no more iodide was forming. This reheating was made by turning back into the first bulb the hydriodic acid which had collected in the flask connected directly with the condenser, and then repeating the process exactly as in the first instance. Much more uniform results were obtained after the slow digestion was introduced, but a white smoke which appeared as a constant factor was not satisfactorily explained.

NO.	AMOUNT OF CHOLINE CHLO- RIDE	AMOUNT OF AgI ON FIRST HEATING	AMOUNT OF CH <sub>3</sub> FOR FIRST HEATING	CH <sub>3</sub> FOR FIRST HEATING	TOTAL CH <sub>3</sub>
	gm.	gm.	gm.	per cent	per cent
1	0.1000	0.4286	0.0274	27.4	35.1
2	0.0431	0.1575	0.01006	23.3	46.3
3	0.0762	0.2907	0.01857	24.4	39.3
4	0.0418	0.1805	0.01153	27.87	32.27
5	0.0311	0.1451	0.00927	29.8	37.3
6	0.0416	0.1964	0.01255	30.01	42.6
7	0.0401	0.1895	0.012105	30.18	40.01
8	0.0655	0.3075	0.01964	29.68	36.99

The slow digestion first used on No. 4 brought about fairly uniform results for the first yield, but although applied also in the succeeding heatings the final yield shows considerable variation. The factor 0.06388 was used to convert the silver iodide into methyl which should amount theoretically to 32.2 per cent of choline chloride. If the hydroxyethyl group is also liberated and calculated as methyl, the total per cent of methyl would be 42.9 per cent.

A mixture of glycerol and choline chloride was then tried. The separation of glycerol was sharp and complete at 112° to 120°, no more precipitate appearing in the silver nitrate solution as the temperature was raised. The second precipitate began to appear usually at 175° and was most abundant at 185°.

NO.		AMOUNT USED	TEMPERA- TURE	WEIGHT OF AgI	CALCULATED TO	
		gm.	C.	gm.	gm.	per cent
1	Glycerol.....	0.1620	112-120°	0.3811	0.1495	Glycerol 92.2
	Choline Cl....	0.1682	180°	0.5607	0.0359	CH <sub>3</sub> 21.3
2	Glycerol.....	0.0719	112-120°	0.1882	0.0738	Glycerol 102.6
	Choline Cl....	0.1000	183°	0.5486	0.0351	CH <sub>3</sub> 35.1
3	Glycerol.....	0.0719	112-120°	0.1782	0.0698	Glycerol 97.2
	Choline Cl....	0.1322	160-243°	0.8927	0.0562	CH <sub>3</sub> 44.2

These figures represent the total yield obtained after several heatings.

With the improved apparatus analyses of the phospholipins were repeated.

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NO.		AMOUNT USED	TEMPERA- TURE	WEIGHT OF Agl		CALCULATED TO	
		gm.	C.	gm.	gm.		per cent
1	Lecithin.....	0.3642	112°	0.1012	0.0397	Glycerol	10.9
			180-185°	0.0766	0.0049	CH <sub>3</sub>	1.34
2	Lecithin.....	0.4927	112°	0.1238	0.0496	Glycerol	10.05
			160-240°	0.1122	0.0072	CH <sub>3</sub>	1.45
3	Egg lecithin...	0.2945	112°	0.0658	0.0258	Glycerol	8.75
			180-190°		0.1065	CH <sub>3</sub>	3.62
4	Lecithin HCl.	0.2868	112°	0.0850	0.0333	Glycerol	11.6
			180-190°	0.2743	0.01752	CH <sub>3</sub>	6.10
5	Kephalin.....	0.1613	112°	0.0338	0.01326	Glycerol	8.21
			180°				
6	Lecithin HCl.	0.1513	112°	0.039	0.1529	Glycerol	9.96
			180-190°	0.3474	0.02219	CH <sub>3</sub>	15.1

These results for glycerol are slightly higher than those obtained by the use of the simple apparatus. If any reliance is to be placed on this method for the determination of the methyl groups, it is evident that lecithin prepared from sheep's brains by the use of acetone, alcohol, and ether contains only one methyl group, the ratio of N : P : CH<sub>3</sub> = 1.89 : 3.73 : 1.45 = 0.12 : 0.09. Koch found, allowing 9.5 per cent glycerol as indicated by the precipitate below 240°, 3.80 per cent for CH<sub>3</sub>, or a ratio of N : P : CH<sub>3</sub> = 1.80 : 3.79 : 3.80 = 1 : 1 : 2. The egg lecithin gives a slightly lower figure for glycerol and a much higher result for methyl, while the lecithin hydrochloride prepared by treatment of the above lecithin with cadmium chloride and removal of the cadmium with hydrogen sulphide as prescribed by Thudichum gives still higher results. The result would indicate for this preparation three methyl groups. Kephalin gave no precipitate at the higher temperature, indicating no methyl groups. A comparison of Nos. 4 and 6 shows widely varying results for samples from the same bottle. This is probably due to the different amounts of time for which each was tested; No. 4 was given two heatings lasting about seven hours, while No. 6 was given three heatings amounting to about ten hours in all. Neither was heated long enough to give final negative results. It is evident that prolonged heating introduces errors—a fact already noticed by Stritar—due to the formation of various alkyl iodides by the hydriodic acid.

These varying results suggested the necessity of testing the method on some of the constituents of lecithin other than choline.

SUBSTANCE	AMOUNT TAKEN gm.	AgI YIELDED gm.	FOUND: CALCULATED:	
			per cent	per cent
Palmitic acid.....	0.1198	0.010 at 190°	0.5 CH <sub>3</sub>	
Oleic acid.....	0.3194	0.0137 " 190°	0.2 CH <sub>3</sub>	
Mono-ethylaminehydro- chloride.....	0.1149	0.0939	9.7 C <sub>2</sub> H <sub>5</sub>	35.6 C <sub>2</sub> H <sub>5</sub>
Trimethyl amine.....	0.0513	0.432	53.79 CH <sub>3</sub>	47.17 CH <sub>3</sub>

A sixth heating of the trimethyl amine hydrochloride gave negative results in the silver nitrate solution. White smoke was a constant accompaniment of the experiment except in the sixth heating, where there was none. Smoke and precipitate began to appear at 150°, a much lower temperature than in the analysis of choline chloride or ethyl amine hydrochloride. The total time of heating was about seventeen hours. It is difficult to account for this excessive yield.

Some methyl iodide was repeatedly fractionated and a portion boiling between 42° to 43° was introduced into a stoppered bottle and dropped into the bulb of the apparatus containing hydriodic acid and given the usual treatment. The precipitate appeared in the silver nitrate solution almost immediately, the stopper having been forced out by expansion. The temperature was, however, raised to 180° and the heating continued till the solution had become clear. 0.2057 gram of methyl iodide yielded 0.3229 gram of silver iodide = 94.84 per cent of the total. Some methyl iodide in a similar stoppered bottle was dropped directly into some alcoholic silver nitrate solution. 0.1505 gram of methyl iodide yielded 0.2440 gram of silver iodide = 97.97 per cent. The analytical method with the apparatus gives then a yield of 96.7 per cent. It is evident from this experiment that the methyl iodide is not lost in transit, but comes over nearly quantitatively, if the direct precipitation be taken as the standard. The only possible explanation for the high yield in the experiment with trimethyl amine hydrochloride would seem to be that hydriodic acid forms unsaturated compounds which yield more than one methyl iodide equivalent. Work by Dr. Nef suggests this as possible and probable.

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From these results it is evident that the method in its present shape is not applicable to the analysis of phospholipins for the purpose of determining their formulae. Hydriodic acid is a powerful reducing agent and attacks both methyl and ethyl groups. Kahan<sup>17</sup> found that methyl iodide heated to 265° for three hours gave no gas, but heated to 270° it went over into methane and ethylene. At 185° methyl iodide is fairly stable, giving only slight evidence of gas. This is not true of ethyl iodide, which goes over into ethane, ethylene, and hydrogen. I found that the ethyl amine hydrochloride gave the sublimate and smoke so characteristic of all the choline chloride determinations. The gas or smoke was collected and found to be an unsaturated hydrocarbon. Goldschmiedt<sup>18</sup> has studied many compounds containing the methyl group in various positions, using this method. He finds that the splitting off of the alkyl group is dependent upon the structure of the compound, the nature and the position of the groups, and that it is impossible quantitatively to differentiate  $N\cdot CH_3$  and  $O\cdot CH_3$ . The methyl group often seems to wander from nitrogen to oxygen. It is possible that longer digestion below the boiling point of hydriodic acid may result in a wider separation by temperature of the methyl and ethyl iodide and admit of a fractional separation of these two alkyl groups.

### SUMMARY.

1. By the use of a paraffin bath, the temperature of which can be controlled, it is possible to obtain by a modified Herzig and Meyer method a sharp separation of the glycerol and alkyl groups in certain phospholipins, the former reacting at 112° and the latter at 180°–190°C.
2. The glycerol is obtained in almost theoretical quantity.
3. The ethyl and methyl groups are not so quantitatively determined and differentiated.
4. Thudichum's preparation of lecithin shows the three methyl

<sup>17</sup> Z. Kahan: *Jour. Chem. Soc.*, xciii, pt. i, p. 132, 1908.

<sup>18</sup> G. Goldschmiedt and A. Kirpal: *Monatschr. f. Chem.*, xvii, p. 491, 1896. G. Goldschmiedt: *ibid.*, xxvii, p. 849, 1906. G. Goldschmiedt and O. Hönigschmid: *ibid.*, xxiv, p. 681, 1903.

groups commonly attributed to lecithin; but our preparations show less.

5. The method shows no methyl or ethyl groups in kephalin.<sup>19</sup>

6. The analysis of synthetic choline chloride shows that hydriodic acid does not split off the methyl groups quantitatively.

7. Ethyl amine hydrochloride gives many of the phenomena, *viz.*, the white smoke and the sublimation in the U tube, noted in the course of the reduction of the choline chloride. These are not so marked in the case of trimethyl amine hydrochloride.

I wish to express here my thanks to Professor A. P. Mathews and to Professor F. C. Koch, to whom I am indebted for many helpful suggestions.

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<sup>19</sup> MacArthur announces in the *Jour. Am. Chem. Soc.*, xxxvi, p. 2397, 1914, that kephalin contains neither choline nor neurine.



## THE INFLUENCE OF THE PLANE OF PROTEIN INTAKE ON GROWTH.<sup>1</sup>

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(Received for publication, January 30, 1915.)

The experience of animal husbandrymen has established the fact that young animals need a relatively high plane of protein intake in order to make the best possible rate of gain. McCollum<sup>2</sup> has shown with pigs that the per cent of the ingested nitrogen retained for growth when the ration contains above 10 per cent of protein is independent of the plane of protein intake. It follows that when the consumption of protein is high, the retention is correspondingly high. In the same paper it was pointed out that although the character of the protein mixture supplied by the cereal grain is such that it can be utilized for growth only to the extent of 20 to 25 per cent, yet growth (retention of nitrogen) continues to take place when the protein content of the ration drawn from wheat or oats was as low as 6.63 to 7 per cent. Lower protein levels were not fed.

Osborne and Mendel<sup>3</sup> have exhibited the curves of growth of rats fed "protein-free milk" with casein to the amount of 4 to 31 per cent of the food mixture. These curves indicate that rats cannot maintain their body weight when less than 12 per cent of casein is fed. With 18 per cent of casein in the diet normal growth was secured, but rapid failure of the animals ensued when 31 per cent of casein was contained in the diet. Their curves show, further, that with 4 per cent of edestin there was steady

<sup>1</sup> Published with the permission of the Director of the Wisconsin Experiment Station.

<sup>2</sup> E. V. McCollum: *this Journal*, xix, p. 323, 1914.

<sup>3</sup> T. B. Osborne and L. B. Mendel: *Ztschr. f. physiol. Chem.*, lxxx, pp. 341-50, 1912.



decline, while with 6.5 per cent of this protein body weight was just maintained. With 9 per cent of edestin a small amount of growth was attained, somewhat better growth with 12 per cent, and normal growth with 18 per cent of edestin. They drew the conclusion that the lowest limit of protein content which can lead to growth is 7 to 9 per cent of the food mixture. We have pointed out that these results are not in harmony with our own observations.<sup>4</sup>

Since it is a matter of great importance to know what plane of protein intake from various sources is essential for body maintenance, and the lowest plane at which the maximum rate of growth is secured, we have undertaken to establish these points with certainty for a variety of foodstuffs.

In our experience even normal growth to the normal adult size and continued maintenance does not necessarily indicate perfect nutrition. Only when the animals reproduce and nourish their young normally, and repeat this at normal intervals, can it be said that the ration is physiologically sufficient.

In the present paper we present curves showing the behavior with respect to growth of rats fed a mixture of skim milk powder (Merrill-Soule), dextrin, and butter-fat. The milk powder content was varied so as to make the protein content of the rations vary between 2 and 10 per cent. For comparison we include also the curves of rats fed with wheat protein (6 per cent), wheat embryo protein (4 per cent), and egg protein (2.45 per cent).

In such experiments as those described it is of the greatest importance that individual records shall not be made the basis of comparison. A number sufficient to exclude the possibility of error, due to individual variations, should be employed. The curves which we present in this paper represent all our experience with the rations described. The similarity of the performance of all the animals of each group is convincing evidence that the results are conclusive. All the experiments were carried out at the same time and in the same room.

<sup>4</sup> E. V. McCollum and M. Davis: *Proc. Am. Soc. Biol. Chemists*, this *Journal*, xiv, p. xl, 1913.

## SUMMARY OF RESULTS.

1. It is evident from the curves shown that the lowest plane of protein intake derived from milk which can maintain young rats without loss of body weight is 3 per cent of the food mixture.

2. There is a progressive increase in the rate of growth with rations derived from milk, as the plane of protein intake is raised between 3 and 8 per cent of the diet.

3. It is evident that for a time at least rats may grow at about half the normal rate when the protein is supplied by the wheat kernel to the extent of 6 per cent of the food mixture.

4. 2.45 per cent of protein derived from desiccated egg is not sufficient to maintain young rats without loss of body weight.

5. During six weeks a ration carrying but 4 per cent of protein from wheat embryo compares favorably with a similar plane of protein intake derived from milk powder, and is somewhat better than 6 per cent of protein from the entire kernel.

6. We believe that this plan of experimentation offers a valuable method of comparison of the proteins from various sources, provided all deficiencies are made up by suitable additions.

## 418 Influence of Protein Intake on Growth

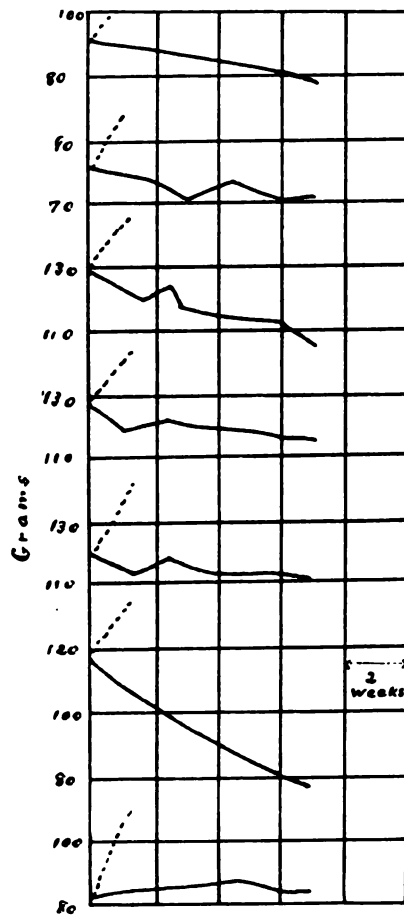


CHART I. (7 males.) Shows a steady loss of body weight in six out of seven of the animals on a diet containing but 2 per cent of milk protein. One has maintained its weight during 49 days on this very low plane of protein intake.

The ration consisted of:

	per cent
Milk powder.....	6.0 (34.0 % N $\times$ 6.25)
Dextrin.....	81.0
Butter-fat.....	5.0
Agar-agar.....	2.0
Salt mixture.....	6.0

### COMPOSITION OF THE SALT MIXTURE

	gm.
NaCl.....	15.00
Na citrate.....	3.70
K <sub>2</sub> HPO <sub>4</sub> .....	34.22
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	0.89
MgSO <sub>4</sub> (anhydrous).....	1.90
Mg citrate.....	7.00
Ca lactate.....	57.02
Fe citrate.....	2.00

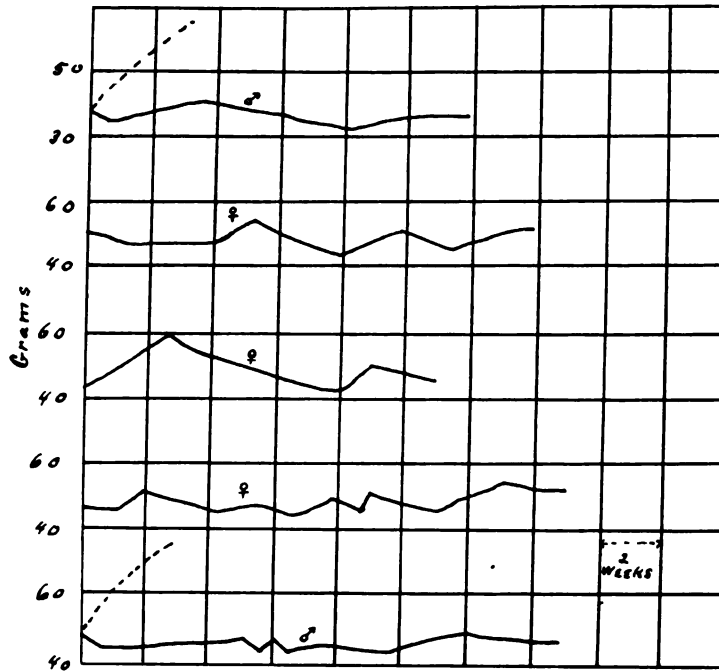


CHART II. (2 males, 3 females.) Shows maintenance of body weight during 100 days with a ration containing 3 per cent of milk protein.

The ration consisted of:

	per cent
Milk powder.....	9.0
Dextrin.....	83.9
Butter-fat.....	5.0
Agar-agar.....	2.0
Fe citrate.....	0.1

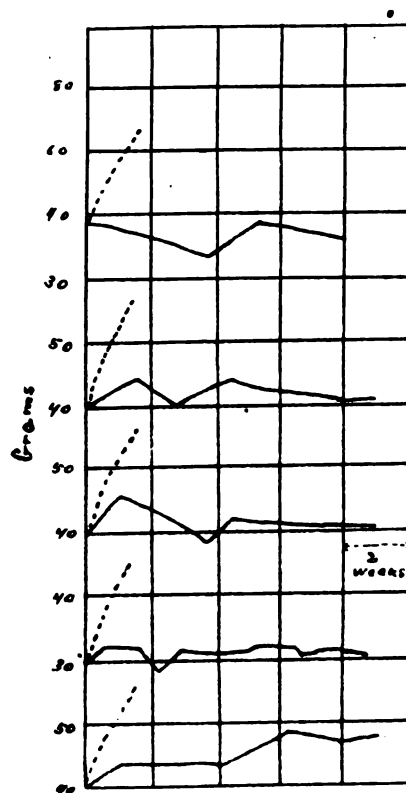


CHART III. (5 males.) Shows the behavior of rats with a ration containing but 3 per cent of protein from milk. The mineral content of the ration in Chart II was derived solely from the 9 per cent of milk powder it contained, and was therefore very low. In order to show whether the low salt content or the protein content was the limiting factor in the ration of this group of animals a salt mixture was added. It is thus made apparent that the low protein content is the limiting factor. The rats behave just as did those without salt addition.

The salt addition consisted of 5 gm. per 100 of ration of the salt mixture fed, the rats receiving 2 per cent of protein of milk (Chart I).



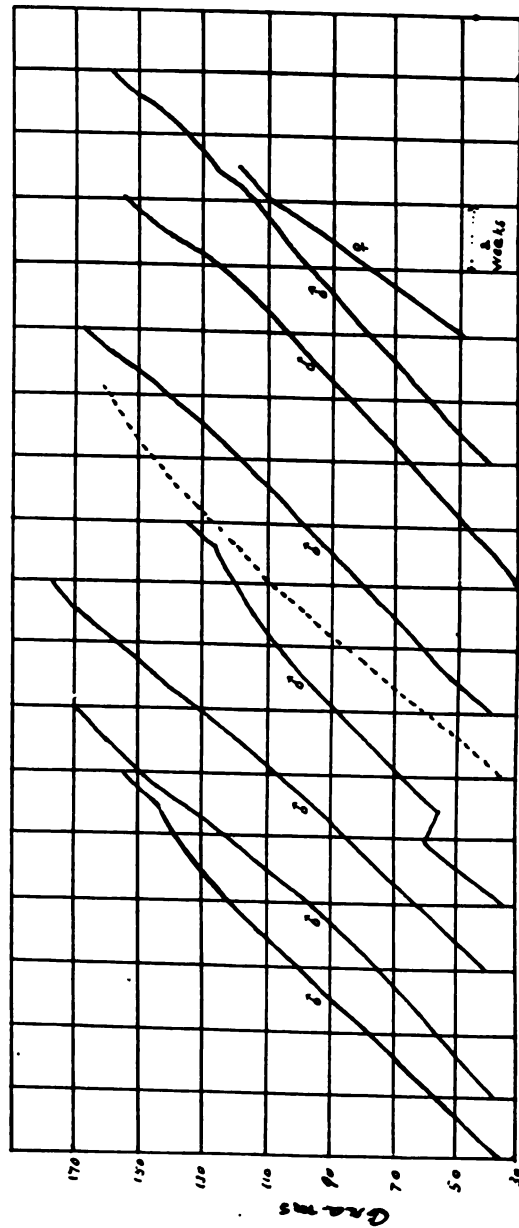


CHART VI. (7 males, 1 female.) Shows how marked is the change in the rate of growth of rats when 6 per cent of milk protein is fed as compared with 5 per cent (Chart V). It is with this plane of protein intake that normal growth is first secured. Dotted line = normal curve.

The ration consisted of:

	per cent
Milk powder .....	18.0
Dextrin .....	75.0
Butter-fat .....	5.0
Agar-agar .....	2.0

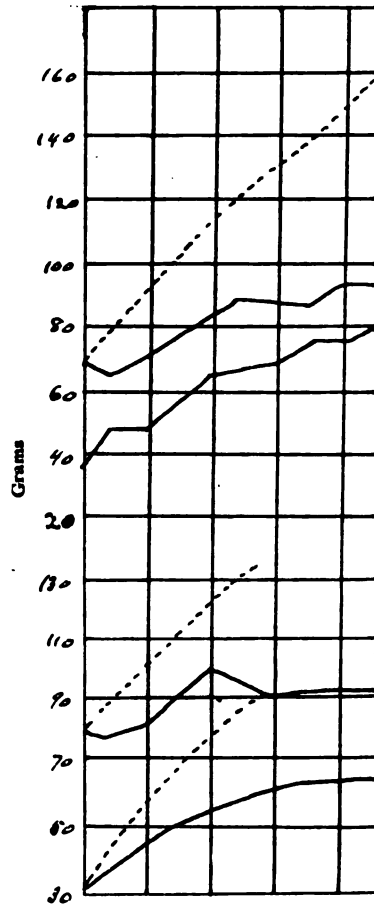


CHART V. (3 males, 2 females)  
Dotted line = normal curve.  
The ration consisted of:

Milk powder.....  
Dextrin.....  
Butter-fat.....  
Agar-agar.....





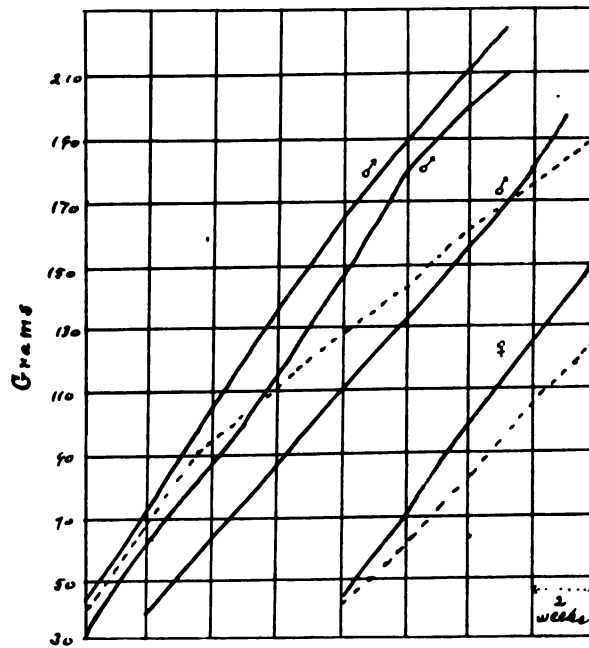


CHART VII. (3 males, 1 female.) Illustrates the growth of rats receiving 8 per cent of milk protein. The growth is in all cases somewhat better than the normal expectation curve. Dotted curve = normal curve.

The ration consisted of:

	per cent
Milk powder.....	24.0
Dextrin.....	69.0
Butter-fat.....	5.0
Agar-agar.....	2.0

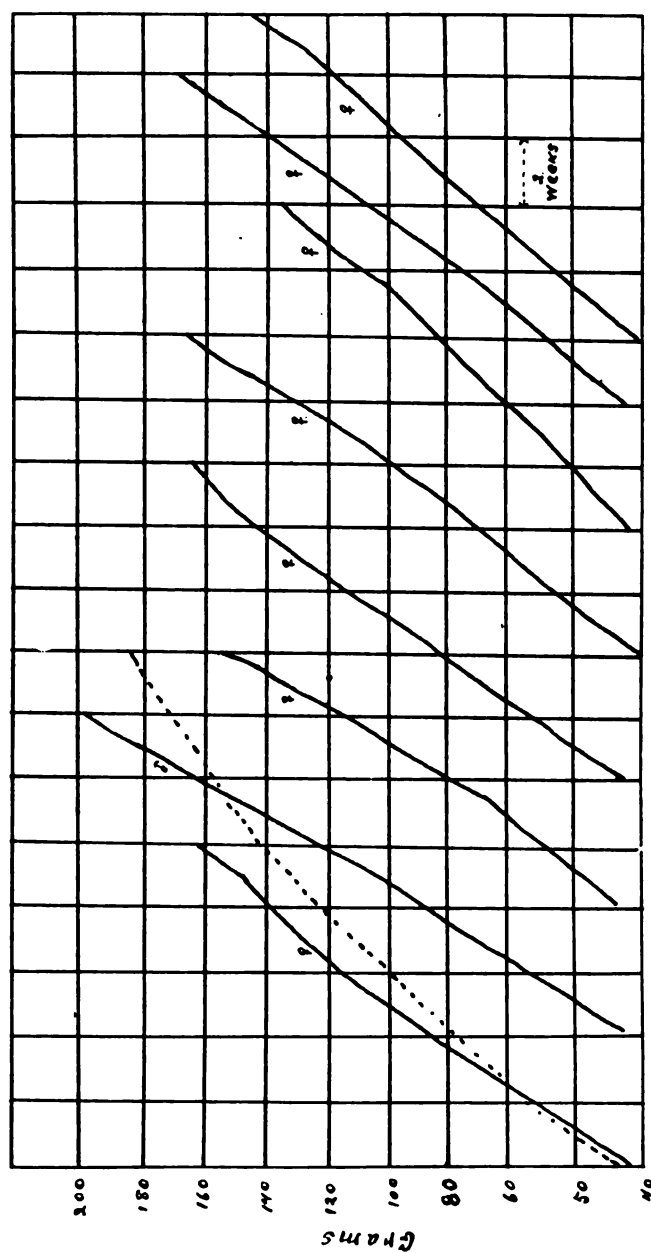


CHART VIII. (7 females, 1 male.) Shows the growth of rats on a diet containing 6 per cent of the proteins of milk, with salt additions. There is no noticeable effect on the rate of growth as compared with the same ration without the salts (Chart VII).

The composition of the ration was as follows:

	per cent
Milk powder.....	24.0
Dextrin.....	67.0
Butter-fat.....	5.0
Agar-agar.....	2.0
Salt mixture.....	3.0

The salt mixture employed had the composition given under Chart I.

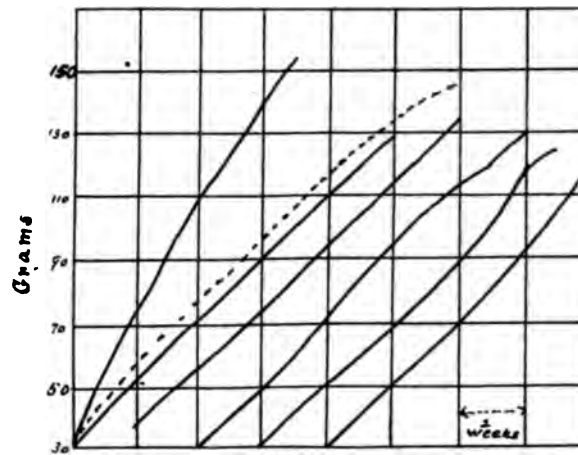


CHART IX. (6 females.) Shows the growth of rats receiving 10 per cent of milk protein. The curves are all those of females. Note the extraordinary growth of one individual.

The composition of the ration was as follows:

	per cent
Milk powder. . . . .	30.0
Dextrin. . . . .	62.9
Butter-fat. . . . .	5.0
Agar-agar . . . . .	2.0
Fe citrate . . . . .	0.1

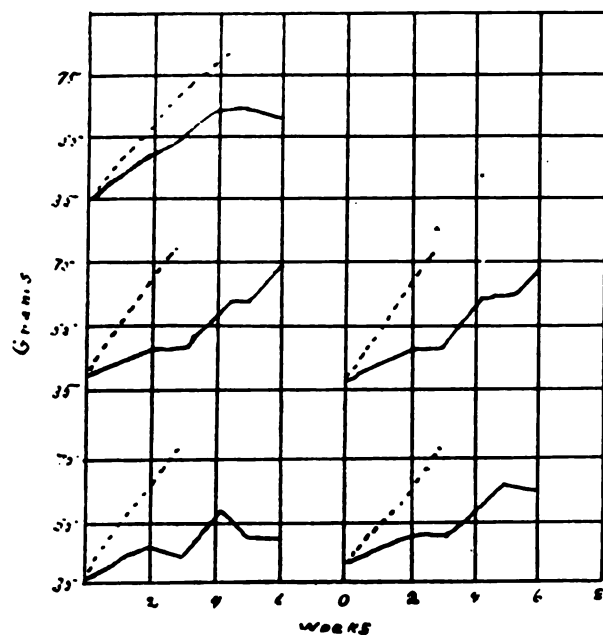


CHART X. (5 males.) Shows the behavior of rats receiving 6 per cent of protein derived from the entire wheat kernel. The rate of growth during the first 42 days is about the same as with 4 per cent milk protein (Chart IV) or 4 per cent wheat embryo protein (Chart XI).

The ration consisted of:

	per cent		gm.
Wheat.....	56.6	NaCl.....	1.40
Dextrin.....	31.5	K <sub>2</sub> HPO <sub>4</sub> .....	2.531
Butter-fat.....	5.0	K citrate.....	0.740
Salt mixture.....	6.9	CaSO <sub>4</sub> 2H <sub>2</sub> O.....	0.578
		Ca lactate.....	7.058

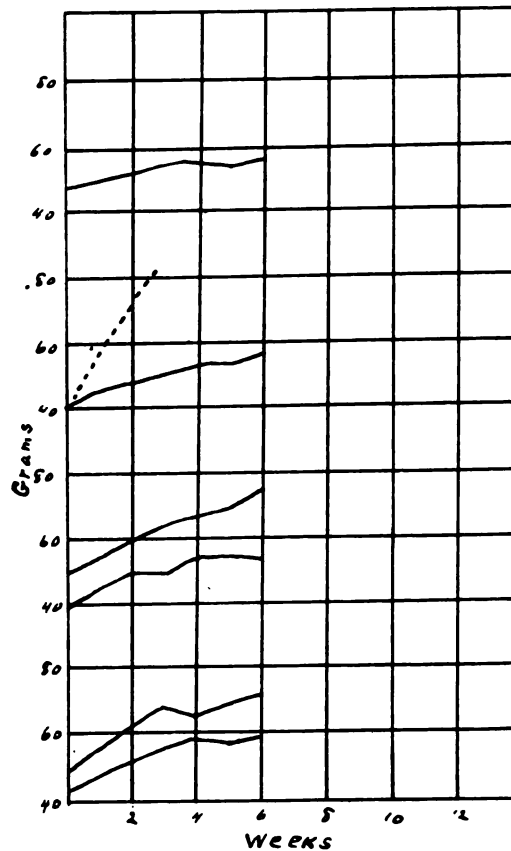


CHART XI. (6 males.) Shows the behavior of young rats receiving but 4 per cent of protein derived from wheat embryo. The growth during the first 42 days is comparable to that of rats getting milk protein at the same level (Chart IV) and with those getting the proteins of the entire wheat kernel 6 per cent (Chart X).

The ration consisted of:

	per cent	COMPOSITION OF THE SALT MIXTURE	
Wheat embryo.....	13.3	NaCl.....	gm. 1.067
Dextrin.....	77.4	K citrate.....	0.205
Butter-fat.....	5.0	K <sub>2</sub> HPO <sub>4</sub> .....	3.016
Salt mixture.....	5.3	CaCl <sub>2</sub> .....	0.386
		CaSO <sub>4</sub> ·2H <sub>2</sub> O.....	0.381
		Ca lactate.....	5.553
		Fe citrate.....	0.100

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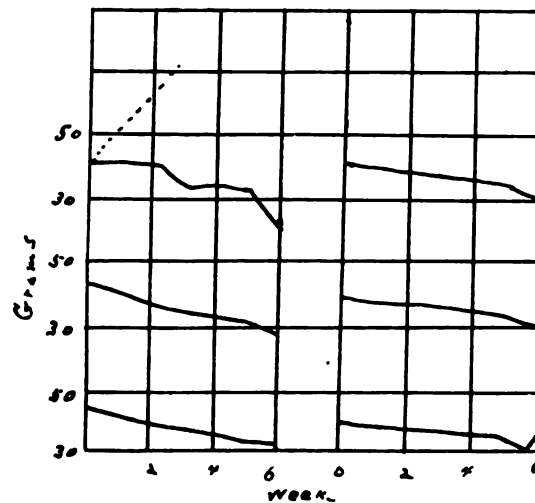


CHART XII. (6 males.) Shows the steady decline of rats receiving 2.53 per cent of protein ( $N \times 6.25$ ) from desiccated egg. Subtracting the nitrogen in the ether extract of egg (0.5 per cent) gives 2.45 per cent as the approximate protein content of the ration.

The ration consisted of:

	per cent
Desiccated egg.....	5.6 (45.31% N $\times$ 6.25)
Dextrin.....	87.4
Butter-fat.....	5.0
Fe citrate.....	0.1

## ON THE MUTAROTATION OF PHENYLOSAZONES OF PENTOSES AND HEXOSES.

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Laboratories of the Rockefeller Institute for Medical Research.)

(Received for publication, January 30, 1915.)

The identification of a sugar can be made unmistakable when it is possible to isolate it in crystalline form, or when there is an abundant supply of material at hand. The biological chemist is often confronted with conditions where a sugar is present in a minimal quantity and where the impurities predominate in a measure which makes the purification of the sugar an impossibility. In such circumstances one is frequently forced to base his opinion on the configuration of the sugar on the properties of the derivative which is always obtainable; namely, on that of the osazone.

Naturally the same osazone is derived from two epimeric sugars, and when an osazone is identified there still remain two possible explanations for the structure of the sugar. The choice between the two can often be made if the information on the constants of the osazone is supplemented by information on the character of the optical rotation of the sugar. In the course of our work on naturally occurring rare sugars we were convinced of the importance of the identification of osazones, and we found that one of the properties which is perhaps the least affected by slight impurities is the character of the mutarotation of the phenylosazone. The initial rotation of an osazone in Neuberg's pyridine-alcohol solution is subject to small variation, dependent on minimal impurities, but the direction of the mutarotation and the equilibrium rotation remains constant.

We found this property of phenylosazones serviceable in the work on the identification of the  $\alpha$ -ketoxylose in a case of pentosuria.<sup>1</sup> Zerner and Waltuch,<sup>2</sup> independently of us and later only

<sup>1</sup> This *Journal*, xviii, p. 319, 1914.

<sup>2</sup> E. Zerner and R. Waltuch: *Monatsh. f. Chem.*, xxxv, p. 1025, 1914.



by a few weeks, called attention to the same property of phenyl-pentosazones. And again we made use of it in the work on chondrosamine.

In view of this we considered it important to determine the character of the mutarotation of the phenylsazones of all the normal pentoses and hexoses. Also the melting points of the osazones were revised on this occasion.

#### EXPERIMENTAL.

##### *Hexoses.*

*Altrose osazone* was prepared from crude altrose obtained by reducing altronic-acid lactone with sodium amalgam. It was recrystallized three times from hot water.

*Gulose osazone* was obtained from gulose prepared by the cyanhydrin synthesis from *l*-xylose. It was twice recrystallized from about 10 per cent acetic acid, dried, and then recrystallized from 4 parts of absolute alcohol plus 10 parts of ether. The product thus purified consists of long, yellow, hair-like needles which do not darken on exposure to air.

*Galactose osazone* was twice recrystallized from a large volume of dilute alcohol and once from absolute alcohol.

*Glucose osazone* was recrystallized from absolute alcohol.

*Corrected melting points of osazones at a rate of heating of about 4 to 5 seconds per 1°.*

OSAZONES	CONTRACTED AT °C.	MELTED AT °C.	DECOMPOSED AT °C.
<i>l</i> -Arabinose.....	160	166	200
<i>d</i> -Xylose.....		164	167
Altrose.....	175	178	189
Gulose.....		168	180
Galactose.....	199*	201	202
Glucose.....		208	208

\* The same sample was heated 1.5 minutes at 190° without melting.

##### *Optical rotations.*

<i>d</i> -Xylosazone. I. Soon after preparation of solution $[\alpha]_D =$ .....	-0.10
After about eighteen hours.....	-0.36
II. The same ten minutes after preparation of solution.....	-0.09
After one hour.....	-0.21
After twenty-four hours.....	-0.43

<i>l</i> -Arabinosazone. Soon after preparation of solution $[\alpha]_D =$ . . . . .	+0.55
After about eighteen hours. . . . .	+0.30
<i>d</i> -Altrose osazone. Soon after preparation of solution $[\alpha]_D =$ . . . . .	-0.40
After twenty-four hours. . . . .	-0.29
<i>d</i> -Gulose osazone. Soon after preparation of solution $[\alpha]_D =$ . . . . .	+0.07
After twenty-four hours. . . . .	+0.50
<i>d</i> -Galactose osazone. I. Soon after preparation of solution $[\alpha]_D =$ . . . . .	+0.73
After eight hours. . . . .	+0.32
II. Soon after preparation of solution. . . . .	+0.80
After twenty-four hours. . . . .	+0.34
<i>d</i> -Glucose osazone. Soon after preparation of solution $[\alpha]_D =$ . . . . .	-0.62
After twenty-four hours. . . . .	-0.35
All determinations were made in a 0.5 dm. tube with D-light, 0.1 gm. of substance in 5 cc. of pyridine-alcohol mixture being used.	



## ON CHONDROITIN SULPHURIC ACID.

### FOURTH PAPER.

BY P. A. LEVENE AND F. B. LA FORGE.

(*From the Laboratories of the Rockefeller Institute for Medical Research.*)

(Received for publication, January 26, 1915.)

In the third paper<sup>1</sup> on this subject observations were reported which led to the conclusion that the nitrogenous component of chondroitin sulphuric acid was a new hexosamine. The data in possession at that time were insufficient to permit a definite conclusion regarding the details of the structure of the new sugar, which was named chondrosamine. Additional information was gained in the course of the present season, and today the structure of the sugar seems to be explained, except on one point; namely, on the relative position of the amino-group attached to the  $\alpha$ -carbon atom.

Chondrosamine was oxidized with bromine to tetroxyaminocaproic acid, which differed from glucosaminic acid in its solubility and in its optical rotation. On reduction with hydriodic acid in the presence of phosphorus, according to Fischer and Tiemann<sup>2</sup> a monohydroxyaminocaproic acid was obtained which seemed to be identical with the substance obtained under the same conditions from glucosaminic acid. Since glucosamine is known to contain a normal carbon chain, it became evident that chondrosamine contained a carbon chain of the same character.

The further data on the structure of the sugar were gained through the study of the products of oxidation of chondrosamine to its dicarboxylic derivative. In the last paper it was stated that on oxidation of desaminochondrosamine with nitric acid a dicarboxylic acid was obtained in the form of its calcium salt. The salt differed from the corresponding salt of isosaccharic acid.

<sup>1</sup> P. A. Levene and F. B. La Forge: *this Journal*, xviii, p. 123, 1914.

<sup>2</sup> E. Fischer and F. Tiemann: *Ber. d. deutsch. chem. Gesellsch.*, xxvii, p. 138, 1894.

I<sub>1</sub> II<sub>1</sub> III<sub>1</sub> IV<sub>1</sub> V<sub>1</sub> VI<sub>1</sub> VII<sub>1</sub> VIII<sub>1</sub>

COOH COOH COOH COOH COOH COOH COOH COOH

H-O H H H H H H H

OHH OH OH OH OH OH OH OH

HOH HOH HOH HOH HOH HOH HOH HOH

H H H H H H H H

COOH COOH COOH COOH COOH COOH COOH COOH

Also here I<sub>1</sub> and III<sub>1</sub>, and VI<sub>1</sub> and VII<sub>1</sub> are identical.

If all these anhydrodicarboxylic acids were known the identification of chondrosic acid should have offered no difficulty. Unfortunately isosaccharic acid was the only known member of this group of acids.

Only two of the six acids or of their anhydro derivatives (mucic and allomucic acids) are optically inactive and can be distinguished by this property from the other acids. It was possible to obtain from chondrosamine an inactive anhydrodicarboxylic acid.

Furthermore, the properties of the osazone<sup>3</sup> derived from chondrosamine (the melting point and character of its optical rotation) seemed identical with those of the osazone obtained from allose or altrose. On the basis of this there seems only one conclusion possible; namely, that chondrosamine has the structure of *l*- $\alpha$ -allosamine. (The osazone of *d*-allose is levorotatory, that of chondrosamine dextrarotatory.) In reality also the structure of *l*- $\alpha$ -altrosamine may be ascribed to chondrosamine. The reasons are the following: Chondrosamine gives rise to the inactive anhydrodicarboxylic acid only under very definite conditions; namely, on treatment of the monocarboxylic amino-acid formed when the amino-sugar is oxidized with bromine, first with nitrous and subsequently with nitric acid. However, if chondrosamine is directly treated with nitrous and then oxidized with nitric acid, the product is an active anhydrodicarboxylic acid.

Evidently in the process of the formation of one of the two anhydro acids the original structure of the sugar suffered a rearrangement. In all probability this rearrangement is brought about by the action of nitrous acid, since there can be derived also two monocarboxylic acids of the desamino chondrosamine. On the basis of this it is permissible to assume that the rearrangement affected the groups attached to the  $\alpha$ -carbon atom, and hence that the two anhydrodicarboxylic acids may be regarded, one as corresponding to altrose and the other to allose.

For the present there exists no experimental evidence for a definite conclusion regarding the arrangement of the groups on the  $\alpha$ -carbon atom in the original sugar.<sup>4</sup>

<sup>3</sup> Levene and La Forge: *loc. cit.*

<sup>4</sup> See J. C. Irvine and A. Hynd: *Jour. Chem. Soc.*, ci, p. 1128, 1912; cv, p. 698, 1914.

An attempt was also made to prepare chondrosaminic acid synthetically by the addition of hydrocyanic acid to ribosimine, but the acid obtained in this manner proved to be different from chondrosaminic acid. Unfortunately the yield was too small to allow a further oxidation into the anhydrodicarboxylic acid. The poor yield of the substance was due principally to our unfamiliarity with the action of prussic acid on ribosimine. The reaction took place with unexpected violence and the products warmed up to a temperature which was detrimental to the compound.

In conclusion it may be mentioned that our observations throw additional light on the relationship of chitonic and chitaric acids. Fischer and Tiemann have shown that glucosamine yields either one or the other of the two monocarboxylic acids, depending on the procedure followed in their preparation, and they have explained the differences of the two acids on the basis of differences in configuration. It was found in the course of this work that also the dicarboxylic acid corresponding to chitaric acid differs from isosaccharic. From the analogy with chondrosamine one may be inclined to regard chitaric<sup>5</sup> and chitonic acids as epimers, one having the configuration of glucose and the other of mannose.

Work on the preparation of other amino-sugars and their oxidation products is now in progress. There are already prepared by us the derivatives of xylose and lyxose.

#### EXPERIMENTAL.

##### *Chondrosaminic acid.*

Sixty grams of chondrosamine hydrochloric acid salt were dissolved in 600 cc. of water and allowed to stand for one week at room temperature with an excess of bromine. The oxidation was then continued for two weeks longer at 35° to 40° with occasional shaking, bromine being added from time to time so that an excess always remained on the bottom of the vessel. The reaction product was then concentrated to a thin syrup in vacuum to remove the excess of bromine and most of the hydrobrominic acid. It was then diluted to about 150 cc. with alcohol,

<sup>5</sup> E. Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxiv, p. 2139, 1891.

which caused the separation of the unchanged amino-hexoses as hydrobromic acid salt. After standing for a few hours in the refrigerator the alcoholic solution was poured off, the residue extracted with a little alcohol, and filtered. The combined alcoholic solutions were then concentrated in vacuum to a small volume, taken up in 400 cc. of water, and the halogen removed first with lead carbonate and finally by warming on the water bath with silver carbonate. The silver and lead were removed from the filtrate with hydrogen sulphide and the solution was warmed for an hour with animal charcoal and filtered. The filtrate, which was of a pinkish color, was then concentrated to a small volume in vacuum when crystallization took place spontaneously. The crystals were washed out of the flask with a small amount of 50 per cent methyl alcohol, filtered with suction, and washed on the funnel with 50 per cent methyl alcohol until nearly colorless. The yield of the crude product, dried in vacuum, was 15 grams; while 4 grams more were obtained by concentrating the mother liquor. About 10 grams of the hexosamine hydrobromic acid salt were recovered from the oxidation product. The substance crystallizes from 10 per cent aqueous solution or from 50 per cent alcohol in short prismatic needles grouped together in rosettes. It has no melting point but darkens slowly above 190°.

0.1396 gm. of substance gave 17.3 cc. amino N, at 22°, 760 mm.

0.0992 gm. of substance gave 0.1326 gm. CO<sub>2</sub> and 0.0568 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>13</sub> O <sub>6</sub> N:	Found:
C.....	36.92	36.61
H.....	6.66	6.44
N.....	7.18	7.02

0.1498 gm. of substance in 2 cc. of 2.5 per cent HCl rotated in a 1 dm. tube with D-light at 28.5°, shortly after preparation of the solution -1.21°; at 25°, after 48 hours -2.19°.

$$[\alpha]_D^{25} = -16.15^\circ, -29.23^\circ$$

*Reduction of chondrosaminic acid with hydriodic acid.*

Two grams of substance were heated with 18 cc. of hydriodic acid, specific gravity 1.96, and 5 grams of phosphonium iodide for four and a half hours in a sealed tube at 100°. The contents of the tube which remained colorless were diluted to 500 cc. with



water and the hydriodic and phosphoric acids removed with lead carbonate. The filtrate which contained halogen was heated on the water bath with silver carbonate, filtered, treated with hydrogen sulphide, filtered, and the filtrate from silver sulphide concentrated in vacuum to dryness. The residue was dissolved in 10 cc. of water and concentrated on the water bath to a thick syrup which solidified on cooling to a semisolid cake. This was extracted with 5 cc. of methyl alcohol, filtered, and dried. The yield was 0.8 gram. For analysis it was dissolved in 2 cc. of water, diluted with 10 cc. of methyl alcohol, filtered from a light flocculent precipitate and concentrated in vacuum to a small volume. The crystals thus formed were filtered with suction and washed with methyl alcohol. The substance contracted at 217° and melted with gas evolution at 222° to 223° (uncorrected).

0.1199 gm. of substance gave 20.5 cc. amino N, at 26°, 763 mm.

0.1026 gm. of substance gave 0.1828 gm. CO<sub>2</sub> and 0.0792 gm. H<sub>2</sub>O.

	Calculated for C <sub>8</sub> H <sub>11</sub> O <sub>5</sub> N:	Found:
C.....	48.98	48.98
H.....	8.84	8.65
N.....	9.52	9.48

#### *Chondrosic acid.*

6.8 grams of the calcium salt described in a previous paper<sup>6</sup> were introduced in portions into 250 cc. of boiling water containing 3.2 grams of oxalic acid. After one-half hour's boiling the solution was treated with a little animal charcoal and filtered, the filtrate concentrated to a syrup which was dissolved in about 30 cc. of acetone, filtered, and again concentrated to a syrup on the water bath. On standing over night large colorless prisms had separated out. Upon completion of crystallization the adhering syrup was dissolved from the crystals with a few cc. of a solution of equal parts of acetone and ether. The crystals were then filtered off with suction and washed with a small amount

<sup>6</sup> Levene and La Forge: *loc. cit.*, p. 128. Instead of allowing the deaminized solution of the hexosamine to stand over night at 42° with nitric acid, we mixed the solution with nitric acid at 0° and allowed it to stand at room temperature. It was then treated as before and we were able to obtain constantly a yield of from 50 to 60 per cent calcium salt.

of the acetone ether solution. The crystals consisted of large parallelopipeds which melted at 179° to 181° (uncorrected) and at this temperature slowly decomposed with gas evolution. The pure substance is rather difficultly soluble in acetone (about 1 to 40 at the boiling point of the solvent). For analysis it was dissolved in acetone, which was allowed to evaporate partially. The melting point of the recrystallized product did not differ from that of the first. The substance is easily soluble in water and alcohol, but nearly insoluble in ether. The yield amounted to 2.2 grams.

0.1094 gm. of substance gave 0.1480 gm. CO<sub>2</sub> and 0.0404 gm. H<sub>2</sub>O.

	Calculated for C <sub>8</sub> H <sub>8</sub> O <sub>7</sub>	Found:
C.....	37.50	37.11
H.....	4.20	4.15

0.1500 gm. of substance required 15.9 cc.  $\frac{N}{10}$  NaOH (calculated: 15.6 cc.).

0.2004 gm. of substance in 3 cc. H<sub>2</sub>O, weight of solution 3.1835 gm., specific gravity 1.0262, rotated in a 1 dm. tube with D-light at 28°, -1.07°, and was unchanged after 5 hours.

$$[\alpha]_D^{25} = -16.56^\circ$$

#### *Epichondrosic acid.*

Fifteen grams of chondrosaminic acid dissolved in 90 cc. of 3.3 per cent hydrochloric acid were deaminized with 15 grams of silver nitrite, first at 0° for two hours and then for twenty hours at room temperature, 4 grams more of silver nitrite being added towards the end of the experiment, together with a few cc. of 10 per cent hydrochloric acid. The excess of silver was removed from the filtrate with hydrogen sulphide and the solution concentrated in vacuum to about 35 cc. An equal volume of concentrated nitric acid was then added in the cold and the mixture evaporated to a thick syrup on a large watch glass in two portions on the water bath. This was taken up in a few cc. of water and again evaporated to a syrup. Crystallization occurred spontaneously during the second evaporation. After standing for a short time the crystals were freed from the syrupy by-products by washing with alcohol and ether mixture. When filtered and dried the yield of the first product amounted to 6.8 grams. It was recrystallized by dissolving in a large amount of acetone and

evaporating the solution to a small volume. Melting point: 201° to 202° (uncorrected).

0.1018 gm. of substance gave 0.1402 gm. CO<sub>2</sub> and 0.0402 gm. H<sub>2</sub>O.

	Calculated for C <sub>4</sub> H <sub>6</sub> O <sub>7</sub> :	Found:
C.....	37.50	37.56
H.....	4.20	4.42

0.1137 gm. of substance required 11.7 cc.  $\frac{N}{10}$  NaOH (calculated: 11.8 cc.).

0.2000 gm. of substance dissolved in 2 cc. of water showed no appreciable rotation in a 1 dm. tube with D-light, measured under conditions where a rotation of 0.02° could not have escaped detection.

*Dehydromucic acid from chondrosic acid.*

One gram of chondrosic acid in 1 cc. of concentrated hydrochloric acid plus 1 cc. of concentrated hydrobromic acid heated in a sealed tube at 150° for eight hours, according to Fischer<sup>5</sup> gave 0.2 gram of dehydromucic acid.

0.1002 gm. of substance gave 0.1684 gm. CO<sub>2</sub> and 0.0276 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> :	Found:
C.....	46.16	45.84
H.....	2.57	3.08

*Pyromucic acid from chondrosic acid.*

One gram of substance was heated at about 200° in a test-tube in an atmosphere of carbon dioxide for one-half hour. The substance sublimed and separated out on the cooler parts of the tube from which it was afterwards removed, extracted with ether, and recrystallized by dissolving in a large amount of ether and allowing the solution to evaporate. It melted at 135°.

0.1000 gm. of substance gave 0.1964 gm. CO<sub>2</sub> and 0.0370 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> :	Found:
C.....	53.57	53.56
H.....	3.57	4.14

*Ribosimine.*<sup>7</sup>

Twenty-five grams of crystalline *d*-ribose were dissolved in about 25 cc. of saturated dry methyl alcoholic ammonia. Crystal-

<sup>7</sup> C. A. Lobry de Bruyn and F. H. Van Leent: *Rec. d. trav. chim. d. Pays-Bas*, xiv, p. 134, 1895.

lization began after two days and was complete in about three days. The hard crystalline crusts were broken up, filtered, and washed with dry methyl alcohol. The yield of dried substance was 22.5 grams. It melted at 137° to 138° (uncorrected) with decomposition.

0.1990 gm. of substance gave 13.4 cc.  $\frac{N}{10}$  HCl (Kjeldahl).

	Calculated for $C_6H_{11}NO_4$	Found:
N.....	9.40	9.42

*Hexosaminic acid from ribose.*

Twenty grams of ribosimine were covered with 10 cc. of water<sup>8</sup> and 6 cc. of 80 per cent hydrocyanic acid added. The reaction proceeded almost violently, and cooling in a freezing mixture had to be resorted to. After about fifteen minutes, the product, a thick brown syrup, was dissolved in about 100 cc. of cold concentrated hydrochloric acid and allowed to stand for twenty-four hours at room temperature. The solution was then concentrated in vacuum to a small volume, diluted, and the ammonia completely removed by distilling in vacuum with an excess of barium hydrate. The barium was removed with a slight excess of sulphuric acid. Lead carbonate was then added to neutral reaction and the precipitates of barium sulphate and lead chloride were filtered off together. The filtrate was warmed with silver carbonate to remove the rest of the halogen, filtered, and the lead and silver were removed from the filtrate with hydrogen sulphide. The solution was then concentrated to a small volume. Upon addition of alcohol a thick brown syrup was precipitated. The supernatant liquid was poured off, and as the syrup failed to crystallize after two weeks' standing, this was extracted several times with about 15 per cent methyl alcoholic hydrochloric acid, and thus a separation from the thick amorphous by-product effected. The alcoholic extract, which was nearly colorless, was concentrated in vacuum to a small volume which was taken up in water and the hydrochloric acid removed by warming on the

<sup>8</sup> As the subsequent experiment shows, this concentration was too great. The experiment should be made in greater dilution, but lack of sufficient ribose prevented a repetition of the experiment for the time being.

water bath with silver carbonate. The silver was removed from the filtrate while still hot and the solution concentrated to a syrup which was treated with a large volume of methyl alcohol. The resulting nearly colorless syrup finally solidified upon standing. The semisolid cake thus obtained was extracted with a few cc. of methyl alcohol and filtered. It was then dissolved in 4 to 5 parts of hot water with the addition of a little animal charcoal and filtered. To the filtrate an equal volume of methyl alcohol was added. On standing the substance crystallized in short prismatic needles grouped together in rosettes. The yield was only about 0.5 gram of the pure substance, which melted with decomposition at 198° (uncorrected).

0.0990 gm. of substance gave 0.1344 gm. CO<sub>2</sub> and 0.0582 gm. H<sub>2</sub>O.

0.0261 gm. of substance gave 3.45 cc. amino N, at 22°, 756 mm. (micro method of Van Slyke).

	Calculated for C <sub>8</sub> H <sub>12</sub> O <sub>6</sub> N:	Found:
C.....	36.92	37.02
H.....	6.66	6.58
N.....	7.18	7.44

0.1500 gm. of substance in 2 cc. 2.5 per cent HCl, weight of solution 2.1663 gm., rotated in a 1 dm. tube with D-light at 23°, after 20 minutes -1.67°; after 24 hours -0.71°; after 48 hours -0.71°.

$$[\alpha]_D^{25} = -22.26^\circ, -9.4^\circ$$

*Epi-isosaccharic acid.*

Ten grams of glucosaminic acid were deaminized with silver nitrite according to Fischer and Tiemann,<sup>9</sup> the resulting solution concentrated to about 25 cc., and an equal volume of nitric acid added in the cold. Oxidation was carried out under the same conditions as already described for chondrosaminic acid. The syrupy product was dissolved in 10 cc. of water, neutralized with strong potassium hydrate solution, and an equal volume of glacial acetic acid added. Upon dilution to 50 cc. with alcohol the acid potassium salt of the dibasic acid soon began to crystallize, and after standing one-half hour in the refrigerator the yield of the substance washed and dried amounted to about 4 grams. For analysis it was recrystallized from about 2 parts of hot water.

<sup>9</sup> Fischer and Tiemann: *loc. cit.*, p. 145.

The product contains 0.5 of a molecule of crystal water, which can be removed in vacuum at 115°.

0.1868 gm. of substance gave 0.0058 gm.  $\text{H}_2\text{O}$ .

0.1811 gm. of dried substance gave 0.0511 gm.  $\text{K}_2\text{CO}_3$ .

	Calculated for $\text{C}_6\text{H}_9\text{O}_5\text{K} + 0.5 \text{H}_2\text{O}$ :	Found:
$\text{H}_2\text{O}$ .....	3.7	3.10
	Calculated for $\text{C}_6\text{H}_9\text{O}_5\text{K}$ :	
K.....	15.73	15.94

*Lead salt of epi-isosaccharic acid.*

2.5 grams of the acid potassium salt were dissolved in about 100 cc. of water and an excess of neutral acetate solution was slowly added in the cold. Crystallization began after a few minutes and was complete after one-half hour. The lead salt crystallizes under these conditions in large, nearly square plates which are very difficultly soluble in water. The yield was practically quantitative. The salt contained 2 molecules of crystal water, which could be removed by heating in vacuum at 150°. The dried preparation analyzed for a neutral lead salt of an anhydrodicarboxylic hexonic acid.

0.2418 gm. of substance gave 0.0204 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_8\text{O}_4\text{Pb} + 2\text{H}_2\text{O}$ :	Calculated for $\text{C}_6\text{H}_6\text{O}_2\text{Pb} + 2\text{H}_2\text{O}$ :	Found:
$\text{H}_2\text{O}$ .....	7.99	8.32	8.40
0.2214 gm. of dried substance gave 0.1690 gm. $\text{PbSO}_4$ .			
	Calculated for $\text{C}_6\text{H}_8\text{O}_4\text{Pb}$ :	Calculated for $\text{C}_6\text{H}_6\text{O}_2\text{Pb}$ :	Found:
Pb.....	49.88	52.18	52.10

7.8 grams of the lead salt were suspended in 150 cc. of water and slightly less than the calculated amount of sulphuric acid was added. The suspension was warmed on the water bath for one hour and then diluted with an equal volume of alcohol, filtered, and the filtrate concentrated in vacuum to a thick syrup. This was dissolved in acetone, filtered, and concentrated on the water bath. The resulting syrup crystallized in the desiccator to a semisolid cake. The crude product was extracted with a small amount of a mixture of 1 part amyl alcohol and 2 parts of ether, filtered, and washed with the same solvent mixture and

finally with dry ether. It may be recrystallized by dissolving in a small amount of acetone and allowing the solution to evaporate nearly to dryness in the air. It crystallizes in aggregates of large plates, the edges of which are usually rounded off by the solvent action of the ether used for washing. The acid is extremely soluble in the usual reagents, with the exception of cold amyl alcohol and ether. Yield, about 2 grams of pure substance. The product contains 1 molecule of crystal water which can be removed by heating *in vacuo* at 78°. The dry substance melts at 160° (uncorrected).

0.1278 gm. of substance gave 0.0107 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> + H <sub>2</sub> O:	Found:
H <sub>2</sub> O.....	8.57	8.38

0.1159 gm. of substance gave 0.1598 gm. CO<sub>2</sub> and 0.0467 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> :	Found:
C.....	37.50	37.57
H.....	4.28	4.47

0.0898 gm. crystal water containing substance required 8.6 cc. <sup>N</sup><sub>10</sub> NaOH (calculated: 8.4 cc.).

0.1637 gm. of dry substance in 2 cc. of H<sub>2</sub>O, specific gravity 1.033, rotated in a 1 dm. tube at 28° with D-light +3.12°.

$$[\alpha]_D^{25} = +39.70$$

## THE BEHAVIOR OF BACTERIA TOWARDS PURIFIED ANIMAL AND VEGETABLE PROTEINS.

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Bacterial decomposition of proteins has been the subject of numerous and extensive investigations. Among those who have made important contributions in this field are Hauser, Salkowski, Hirschler, Nencki, Winternitz, Wernick, Salus, Stockley, Bienstock, and Tissier. Little attention has been given, however, to the decomposition of pure proteins in media from which all other nitrogen-containing substances were excluded.

In the painstaking investigations of Bainbridge<sup>1</sup> sufficient evidence was acquired to indicate that certain aerobic and facultative anaerobic bacteria are in themselves unable to initiate or bring about the decomposition of purified unaltered animal proteins. In other words, it was shown that media which contain native proteins as the only nitrogenous ingredients fail to furnish the necessary conditions of bacterial development, although all the other requirements of a culture medium are satisfied. The investigations of Bainbridge were limited to a relatively small number of bacteria, not including the so called "putrefaction organisms," and the proteins egg albumin and serum albumin alone were employed.

The present investigation is in part a repetition of the work of Bainbridge. In addition, however, considerable attention was devoted to putrefactive anaerobes of the *putrificus* and *malignant edema* bacillus type. Furthermore, the deportment of bacteria towards the vegetable protein edestin was studied; and, finally, a number of experiments were conducted with a view of determining the rôle of bacterial enzymes in the so called "proteolytic activities" of bacteria in nature.

<sup>1</sup> F. A. Bainbridge: *Jour. Hyg.*, xi, p. 341, 1911.



Crystallized egg albumin was prepared by the method of Hopkins and Pinkus.<sup>2</sup> After two or three recrystallizations and at least ten precipitations with powdered sodium sulphate, the albumin solution was subjected to dialysis until the dialysate ceased to give a reaction for ammonia with Nessler's reagent. This required from five to seven days.

The per cent of nitrogen present in the dialyzed albumin solution was determined by the Kjeldahl method, and from this the amount of protein was estimated by multiplying the nitrogen figure by 6.25. The protein solution was then diluted with ammonia-free water until it contained 0.25 to 0.5 per cent protein. The following inorganic salts were added: sodium chloride 0.5 per cent, sodium sulphate 0.1 to 0.2 per cent, calcium chloride 0.1 per cent, and potassium phosphate 0.1 per cent. The final reaction was adjusted so that the solution was faintly acid to litmus.

Sterilization of the pure-protein medium was accomplished by Berkefeld filtration, which appears to be the only method that will insure the albumin against any action that may change its chemical composition. The Berkefeld filter and the receiving bottle were sterilized by heating in an autoclave for one hour under an extra pressure of ten to twelve pounds. The sterile filtrate was introduced in accurately measured quantities into sterile test-tubes and flasks. Ten cc. of the albumin solutions were transferred to the test-tubes, while the flasks received 25 cc. Sterility tests were always made by streaking slant agar tubes with several loopfulls of the solutions and incubating the agar for 24 hours at 37°C. and for an additional period of 24 to 36 hours at 24°C.

The sterile solutions of albumin and inorganic salts were inoculated with 24 hour slant agar cultures of the various organisms. An extremely small amount of the growth was removed from the surface of the agar with a platinum needle, great care being exercised to prevent the transfer of any of the agar. After thoroughly shaking the test-tubes or flasks definite dilutions were made as follows: One cc. of the bacterial suspension was mixed with 100 cc. of sterile water in dilution bottles. This constituted dilution A (1:100). One cc. of dilution A was further diluted by mixing it with 100 cc. of water (dilution B, 1:10,000). Agar plates were poured with 0.5 cc. of the last dilution. Duplicate series of plates were always prepared, and the results usually averaged. The plates were incubated at 24° or 37°C., according to the organism under observation. Counting of the colonies was done on the Wolfhügel apparatus, with the help of a magnifier.

The technique employed in the preparation of the solutions of serum albumin was essentially the same as that already described for egg albumin. Owing to the difficulty of obtaining fresh horse serum or serum that was free from chemical preservatives, the number of tests made with serum albumin was comparatively small. Furthermore, the crystallization of serum albumin was attended with considerably more difficulty than that of the egg albumin.

<sup>2</sup> F. G. Hopkins and S. N. Pinkus: *Jour. Physiol.*, xxiii, pp. 130-36, 1898-99.

Edestin was prepared from hemp-seed, as described by Osborne.<sup>3</sup> After repeated dialysis the precipitate in the dialyzer was collected on filter paper, washed, and introduced into a flask containing ammonia-free water. The amount of protein present in the suspension after thorough shaking was determined by the Kjeldahl method.

A definite portion of the edestin suspension was sufficiently diluted with ammonia-free water to reduce the edestin content to 0.5 to 1.0 per cent. Sodium chloride (0.5 per cent) and sodium sulphate (0.2 per cent) were added, and the medium was rendered faintly alkaline to litmus paper with a very weak solution of sodium hydroxide. Complete solution was brought about by the addition of the alkali, yet the alkalinity was so slight as to offer little, if any, resistance to bacterial development. Sterilization was effected by Berkefeld filtration, as in the preparation of egg and serum albumin solutions. Sterile calcium chloride and potassium phosphate were added to the filtered solution in requisite amounts (0.1 per cent). These two agents were added after the filtration so as to avoid any interference of them with the filtration, and to prevent the loss of calcium phosphate from the medium.

The final solution containing the purified edestin was transferred to test-tubes and was tested for sterility before being inoculated. Coagulation tests were not made with the edestin solutions, but the same quantitative bacterial determinations on agar plates were made as in the study of the deportment of bacteria in solutions containing purified animal albumin.

*The behavior of aërobes and facultative anaërobes in solutions containing purified egg albumin, serum albumin, and the vegetable protein edestin as the only available sources of nitrogen.*

The following organisms were employed:

<i>B. subtilis</i>	<i>Bacillus Z</i> (a rapid gelatin liquefier
<i>B. anthracis</i>	resembling <i>B. proteus vulgaris</i> )
<i>B. pyocyaneus</i>	<i>B. coli communis</i>
<i>B. prodigiosus</i>	<i>B. typhi</i> (two strains)
<i>B. proteus vulgaris</i> (two strains)	<i>B. pullorum</i> (two strains)
<i>B. proteus mirabilis</i>	<i>S. pyogenes aureus</i> .

The figures in the accompanying tables (I, III, and IV) are the averages obtained from duplicate sets of plates which were prepared from the inoculated media at definite intervals following the inoculation. The small numbers of colonies of *B. subtilis* and *B. anthracis* that were obtained on the agar plates may be attributed largely to the fact that both of these organisms form

<sup>3</sup> T. B. Osborne: *Abderhaldens Handb. d. biochem. Arbeitsmethoden*, ii, p. 289, 1910.

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TABLE I.

Showing the behavior of bacteria in a medium containing pure egg albumin.  
Composition of medium: egg albumin 0.2 to 0.8 per cent, sodium chloride 0.5 per cent, sodium sulphate 0.2 per cent, calcium chloride 0.1 per cent, and acid potassium phosphate 0.1 per cent.

ORGANISMS	NO. OF COLONIES DEVELOPING ON AGAR PLATES. AVERAGES. (FINAL DILUTION OF ORIGINAL SUSPENSION 1:10,000)						
	Pouring of plates after inoculation of albumin solution						
	Imme- diately	24 hrs.	48 hrs.	72 hrs.	4-6 dys.	1-2 wks.	3 wks.
<i>B. subtilis</i> .....	176	—	76	—	—	16	—
<i>B. subtilis</i> .....	1	1	—	9	—	1	10
<i>B. subtilis</i> .....	0	1	—	0	—	2	—
<i>B. subtilis</i> .....	1	0	0	—	—	—	—
<i>B. anthracis</i> .....	0	2	—	1	—	4	—
<i>B. anthracis</i> .....	1	2	3	—	—	3	—
<i>B. anthracis</i> .....	0	4	—	—	—	1	—
<i>B. pyocyaneus</i> .....	398	225	—	785	—	976	1518
<i>B. pyocyaneus</i> .....	5	368	—	488	—	68	—
<i>B. pyocyaneus</i> .....	125	696	—	—	1209	—	—
<i>B. pyocyaneus</i> .....	58	8	—	—	53	—	—
<i>B. prodigiosus</i> .....	126	492	—	166	—	567	—
<i>B. proteus vulgaris</i> (1).....	1	0	0	—	—	0	—
<i>B. proteus vulgaris</i> (1).....	16	4	0	—	—	0	—
<i>B. proteus vulgaris</i> (2).....	83	2	—	—	—	0	—
<i>B. proteus mirabilis</i> .....	1	1	0	—	—	0	—
<i>Bacillus Z</i> .....	290	1215	—	—	2809	—	—
<i>Bacillus Z</i> .....	15	1467	—	—	too many to count	0	0
<i>Bacillus Z</i> .....	328	825	—	—	—	443	—
<i>B. coli communis</i> .....	count- less	457	501	—	103	181	—
<i>B. coli communis</i> .....	494	1062	—	—	—	68	—
<i>B. typhi</i> (1).....	23	120	—	177	—	42	17
<i>B. typhi</i> (2).....	540	113	—	117	—	—	—
<i>B. typhi</i> (2).....	166	5	—	—	—	5	—
<i>B. typhi</i> (2).....	330	20	—	—	—	44	—

TABLE I—*Concluded.*

ORGANISMS	NO. OF COLONIES DEVELOPING ON AGAR PLATES. AVERAGES. (FINAL DILUTION OF ORIGINAL SUSPENSION 1:10,000)						
	Pouring of plates after inoculation of albumin solution						
	Imme- diately	24 hrs.	48 hrs.	72 hrs.	4-6 dys.	1-2 wks.	3 wks.
<i>B. pullorum</i> (1).....	89	211	—	—	—	9	—
<i>B. pullorum</i> (2).....	3	9	—	—	—	0	—
<i>S. pyogenes aureus</i> .....	27	65	—	11	—	0	—
<i>S. pyogenes aureus</i> .....	165	58	—	3	—	1	—
<i>S. pyogenes aureus</i> .....	154	135	—	—	2	—	—
<i>S. pyogenes aureus</i> .....	84	23	12	—	—	0	—

clumps in culture media and that a thorough distribution of them in the agar at the time of pouring was impossible. In a few instances the first set of plates showed no colonies, while, with one exception, in subsequent sets some colonies appeared. Such irregularities must be expected with these organisms.

Since the results are clearly set forth in the tables, it is unnecessary to comment on them at great length. It will be seen at a glance that little bacterial development took place in any of the pure-protein media. Control experiments in which bouillon was inoculated at the same time and in the same manner as the albumin and edestin solutions were conducted. Furthermore, cultural tests were made with solutions containing a small amount (0.1 to 0.2 per cent) of Witte's peptone, along with the regular ingredients of the pure-protein media. In both cases bacterial development was rapid and the organisms multiplied to such an extent as to be apparent to the unaided eye, by the turbidity produced. Odor and microscopic tests further substantiated this fact.

While in some of the experiments an appreciable increase is registered in the number of colonies after the first plate pouring, the difference is usually offset by a decrease in other tests with the same bacteria. This is particularly true of *B. pyocyaneus* and *Bacillus Z.* The poor showing that is made by *B. proteus vulgaris*, in the test medium is perhaps most surprising. This organism, which has frequently been associated with rapid proteolysis and even putrefaction, is very quickly reduced in numbers,

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TABLE II.

Control experiments showing the behavior of bacteria in a nitrogen-free medium containing the following inorganic salts: sodium chloride 0.5 per cent, sodium sulphate 0.2 per cent, calcium chloride 0.1 per cent, and acid potassium phosphate 0.1 per cent.

ORGANISMS	NO. OF COLONIES DEVELOPING ON AGAR PLATES. AVERAGES. (FINAL DILUTION OF SUSPENSION 1:20,000)					
	Pouring of plates after inoculation of the salt medium					
	Imme- diately	24 hrs.	48 hrs.	72 hrs.	4-6 dys.	1-2 wks.
<i>B. subtilis</i> .....	0	0	—	0	—	0
<i>B. subtilis</i> .....	134	12	—	—	Very few <i>subtilis</i> colonies. Plates con- taminated.	—
<i>B. subtilis</i> .....	1	0	—	—	0	—
<i>B. subtilis</i> .....	1	0	0	—	—	—
<i>B. anthracis</i> .....	2	5	1	—	—	3
<i>B. anthracis</i> .....	4	5	—	—	—	6
<i>B. pyocyaneus</i> .....	215	589	—	37	—	71
<i>B. pyocyaneus</i> .....	6	411	—	—	237	—
<i>B. pyocyaneus</i> .....	244	15	—	—	622	—
<i>B. proteus vulgaris</i> .....	0	0	0	—	—	0
<i>B. proteus mirabilis</i> .....	3	0	0	—	—	0
<i>Bacillus Z</i> .....	41	75	—	—	67	—
<i>Bacillus Z</i> .....	397	201	—	—	—	134
<i>B. typhi</i> .....	368	25	—	123	—	—
<i>S. pyogenes aureus</i> .....	40	7	—	0	—	0
<i>S. pyogenes aureus</i> .....	44	16	—	—	0	—
<i>S. pyogenes aureus</i> .....	169	0	0	—	—	0

and disappears within forty-eight hours after the inoculation. The inhibiting influence is even more pronounced than that of the control medium of inorganic salts (Table II). *Staphylococcus aureus* also undergoes rapid depletion in each of the pure-protein media. *B. coli* and *B. typhi* suffered marked and progressive decreases except in the medium containing the pure-

protein edestin, in which *B. coli* made slight but insignificant progress.

The edestin medium was also somewhat less inhibitive or non-supporting than the egg or serum albumin for *B. subtilis*, *B. anthracis*, and *B. pyocyaneus*. The differences were but unimportant, however, and hence it may be safely stated that this

TABLE III.

Showing the behavior of bacteria in solutions of serum albumin. Composition of medium: serum albumin 0.2 per cent, sodium chloride 0.5 per cent, sodium sulphate 0.2 per cent, calcium chloride 0.1 per cent, and potassium phosphate 0.1 per cent.

ORGANISMS	NO. OF COLONIES ON AGAR PLATES. AVERAGES. (FINAL DILUTION OF BACTERIAL SUSPENSION 1:20,000)		
	Pouring of plates after inoculation of medium		
	Immediately	24 hrs.	48 hrs.
<i>B. subtilis</i> .....	11	25	20
<i>B. subtilis</i> .....	17	23	30
<i>B. anthracis</i> .....	2	14	15
<i>B. anthracis</i> .....	2	19	16
<i>B. pyocyaneus</i> .....	2366	2479	1971
<i>B. pyocyaneus</i> .....	1801	2709	1582
<i>B. proteus vulgaris</i> (1).....	69	3	1
<i>B. proteus vulgaris</i> (2).....	50	3	1
<i>Bacillus Z</i> .....	379	1675	1373
<i>Bacillus Z</i> .....	522	2440	945
<i>B. coli communis</i> .....	185	718	492
<i>B. coli communis</i> .....	185	749	542

vegetable globulin, like egg and serum albumin, resists direct decomposition by the microorganisms employed.

The failure to develop perceptively in these pure-protein media undoubtedly lies in the fact that the bacteria are not supplied with nitrogen which is available for their immediate use. In other words, they come to a standstill in their development or they die from inanition. This view is supported by the control experiments (Table II) in which a medium consisting purely of

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inorganic salts is used, and in which the results are practically the same as in the experiments involving the use of the same salts plus the pure protein (Tables I, III, and IV); and it is made all the more certain by the following experiments. A medium containing the ingredients of Uschinsky's medium, together with

TABLE IV.

Showing the behavior of bacteria in solutions containing edestin. Composition of medium: edestin 0.5 to 1.0 per cent, sodium chloride 0.5 per cent, sodium sulphate 0.2 per cent, calcium chloride 0.1 per cent, and potassium phosphate 0.1 per cent.

ORGANISMS	NO. OF COLONIES DEVELOPING ON AGAR PLATES. AVERAGES						
	Pouring of plates after inoculation of edestin solutions						
	Imme- diately	24 hrs.	48 hrs.	72 hrs.	4-6 dys.	1-2 wks.	3 wks.
<i>B. subtilis</i> .....	2	17	62	—	—	—	—
<i>B. anthracis</i> .....	1	0	28	—	—	—	—
<i>B. anthracis</i> .....	26	25	37	—	—	33	—
<i>B. anthracis</i> .....	11	6	4	—	—	1	—
<i>B. pyocyaneus</i> .....	1678	2453	—	—	—	4346	—
<i>B. pyocyaneus</i> .....	617	2903	4391	—	—	5115	—
<i>B. prodigiosus</i> .....	769	1313	1940	—	—	—	—
<i>B. prodigiosus</i> .....	308	937	1593	—	—	—	—
<i>B. proteus vulgaris</i> (1).....	4	0	0	—	—	—	—
<i>B. proteus vulgaris</i> (1).....	0	0	0	—	—	—	0
<i>B. proteus vulgaris</i> (2).....	1	7	0	—	—	0	—
<i>Bacillus Z</i> .....	1099	2248	2072	—	—	352	—
<i>B. coli communis</i> .....	1042	964	734	—	—	—	—
<i>B. coli communis</i> .....	1086	2312	—	—	—	2	—
<i>S. pyogenes aureus</i> .....	1051	1253	—	—	—	—	10

0.5 per cent pure edestin, was inoculated with the different test organisms and incubated for definite periods. Bacterial development was so rapid that in comparatively few hours the medium became clouded, and the bacteria were too numerous for determination by the plate method.

*Coagulation tests for unchanged proteins.*

In order to obtain still more complete evidence as to whether bacteria are in themselves able to attack native proteins, experiments were conducted which involved the estimation of coagulable protein after different periods of incubation following the inoculation of the pure-protein medium. The method employed was as follows: Flasks containing 25 cc. of the test egg albumin solution were inoculated with *B. anthracis*, *B. proteus vulgaris*, and *Bacillus Z*. After an incubation period of from sixteen to

TABLE V.

*Showing the results of coagulation tests for unchanged proteins. Composition of protein medium: pure egg albumin 0.8 per cent, sodium chloride 0.5 per cent, sodium sulphate 0.2 per cent, calcium chloride 0.1 per cent, and potassium phosphate 0.1 per cent. 25 cc. of medium in each flask.*

NO. OF FLASK	ORGANISMS	INCUBATION PERIOD	WEIGHT OF COAGULATED PROTEIN AT END OF INCUBATION PERIOD	
		days.	gm.	
3	None (Control).....	16	0.0819	
4	None (Control).....	16	0.0815	
5	<i>Bacillus Z</i> .....	19	0.0560	Medium contaminated.
6	<i>Bacillus Z</i> .....	19	0.0679	Medium contaminated.
7	<i>Bacillus Z</i> .....	17	0.0819	
8	<i>Bacillus Z</i> .....	17	0.0815	
9	<i>B. proteus vulgaris</i> .....	18	0.0856	
10	<i>B. proteus vulgaris</i> .....	18	0.0829	
11	<i>B. anthracis</i> .....	23	0.0784	
12	<i>B. anthracis</i> .....	23	0.0835	

twenty-three days the contents of the flasks were made faintly acid with acetic acid and heated in a water bath (about 99°C.) for fifteen to twenty minutes. The coagulum was collected in a weighed Gooch crucible containing a thin asbestos mat, and dried to constant weight.

The results as given in Table V show that there was no real loss of coagulable protein, barring two exceptions. Two duplicate flasks which were inoculated with *Bacillus Z* registered a loss of normal protein, the one about 17 per cent and the other 31.6 per cent. Both of these flasks were badly contaminated, which fact undoubtedly offers an explanation of the partial



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decomposition of the proteins. The associated bacteria were present in sufficient numbers to furnish available nitrogen, either directly or indirectly, for the test organism and the development of a requisite amount of enzyme to bring about proteolysis. Further experiments with *Bacillus Z* in which contamination was excluded showed no decomposition whatever of the egg albumin. These results are in perfect accord with those of Bainbridge, and substantiate those which were obtained earlier in our investigation.

### *The behavior of the putrefactive anaërobes towards pure animal and vegetable proteins.*

The meaning of the term putrefaction and the organisms which cause putrefactive decomposition have been the subject of so much discussion in recent years that a brief reference to some of the opinions and theories can not appear out of place here.

In the controversies two important views of the biology and chemistry of putrefaction have been strongly defended. According to the older theory of Hauser and many other investigators, and the one still held by Tissier<sup>4</sup> and Martelly, Metchnikoff,<sup>5</sup> and others, putrefaction is caused not only by obligate anaërobes, but by facultative aërobes, and even a limited number of obligate aërobes. On the other hand, it is claimed by Bienstock<sup>6</sup> and Rettger<sup>7</sup> that real putrefaction is the work of certain anaërobes only. In an article published quite recently Tissier is loath to give up his views, and writes: "Comme il est facile de le voir . . . les proteolytiques les plus puissants sont des bacteries anaërobies stricts. Ce ne sont pas les seules, comme le croyait Bienstock; avec Martelly, nous avons vu qu'il y a des aërobes facultatifs capables de jouer le même rôle, mais leur action est loin d'être aussi rapide."

An organism to which Tissier and Martelly attached special significance is the facultative anaërobe, *B. proteus vulgaris*. In

<sup>4</sup> H. Tissier and Martelly: *Ann. de l'Inst. Pasteur*, xvi, p. 865, 1902.  
H. Tissier: *ibid.*, xxvi, pp. 522-29, 1912.

<sup>5</sup> E. Metchnikoff: *ibid.*, xxii, p. 928, 1908.

<sup>6</sup> Bienstock: *Arch. f. Hyg.*, xxxvi, pp. 335-89, 1899; xxxix, p. 390, 1901.

<sup>7</sup> L. F. Rettger: this *Journal*, ii, pp. 71-86, 1906-07; iv, pp. 45-55, 1908.

their study of the *proteus* group of bacteria, with special reference to its ability to initiate putrefactive changes, Rettger and Newell<sup>8</sup> employed twenty-five different strains of *proteus* organisms, four of which came from the Pasteur Institute of Paris. In no instance could putrefaction be brought about through the agency of these organisms; nor was there any indication whatever of the decomposition of the special egg-meat medium by any member of this group when the tests were made under anaërobic conditions. Over half of the twenty-five strains of bacteria were those of *proteus vulgaris*, which has always been regarded as the most active proteolytic organism in the group.

Representatives of the *proteus* group are frequently present in putrefactive mixtures, as Tissier and others showed; but, while they may aid materially in the ultimate decomposition of the intermediate products of putrefaction, their ability to initiate real putrefactive changes is far from being established. According to Rettger the products of real putrefaction include hydrogen sulphide, mercaptan, and aromatic oxyacids, besides the usual products of tryptic digestion or proteolysis; namely, leucine, tyrosine, and tryptophane. No mention is made by Tissier of the presence of mercaptan in any of his so called putrefaction tests with members of the *proteus* group.

While none of the strict aërobes or facultative anaërobes used in the present investigation were able to utilize the pure animal and vegetable proteins without the aid of some intermediary agent, it seemed highly probable that the putrefactive anaërobes could do so. It should be stated, however, that in earlier investigations<sup>9</sup> of these bacteria, and their deportment towards normal egg-white, no evidence was obtained that these organisms could decompose the egg albumin or even develop in the egg-white. The egg-white even appeared to have an antiseptic, and in a measure bactericidal, action on the anaërobes. This property was undoubtedly not due to the egg albumin, but to other agencies in the egg.

The anaërobes which were employed in the present investigation were the well known putrefactive organisms, *B. putrificus*.

<sup>8</sup> L. F. Rettger and C. R. Newell: *ibid.*, xiii, p. 342, 1912-13.

<sup>9</sup> L. F. Rettger and J. A. Sperry: *Jour. Med. Research*, xxvi, pp. 55-64, 1912.

*B. anthracis* *symplasmatica*, and *B. cereus* *midway*. The organisms were grown continuously in the egg-meat medium recommended by one<sup>10</sup> of us. Anaerobiosis was effected by the Wright and the Brothner pyrogallol methods. Tubes containing sterile egg-meat medium were heated at 50°C. for a few minutes, in order to expel the free oxygen. They were then inoculated with the anaerobe to be investigated, the conditions made anaerobic, and the tubes incubated for four days at 37°C. At the end of this period there was usually marked putrefaction, as indicated by the partial digestion of the solid medium and by the characteristic odor of putrefaction. Slant agar tubes were inoculated with two or three loopfulls of the medium taken from the watery layer. The agar tubes were incubated anaerobic for three to four days. The resultant surface growths were employed for the inoculation of the pure-protein test media.

Solutions of egg albumin and of edestin were inoculated with minute quantities of the agar surface growth in such a way as to prevent the transfer of any of the culture medium itself. Tubes of plain bouillon were inoculated at the same time for controls. The egg albumin tests were made with twenty-three different tubes, twelve bouillon controls being used. They were examined at the end of 5, 7, 10, and 16 days. In the experiments on edestin ten edestin tubes and four controls were employed, and the examinations were made after intervals of 4, 14, and 19 days, and 3 weeks.

In every instance the control tubes gave marked evidence of growth with typical putrefactive odor. All the egg albumin and edestin tubes remained perfectly clear and odorless. Microscopic examinations of Gram-stained films prepared from the different media showed that the plain bouillon tubes contained large numbers of the putrefactive bacteria, while but few were present in the mounts made from the albumin or edestin medium, although all the tubes had been inoculated with as nearly the same number of organisms as was possible. Transplantations made from the different sets of tubes to egg-meat medium resulted in the characteristic putrefactive decomposition in the time usually required.

While quantitative coagulation tests were not made, tubes of the egg albumin and the edestin medium were heated as previously

<sup>10</sup> Rettger: this *Journal*, ii, pp. 71-86, 1906-07.

described, after definite periods of incubation, and the amount of coagulable protein compared with that originally present. No differences could be noted.

The above results seemingly justify the conclusion that even the putrefactive anaërobes, *B. putrificus*, *B. anthracis symptomatici*, and *B. edematis maligni*, are in themselves unable to bring about decomposition of native proteins, particularly egg albumin and edestin. In order to determine whether these organisms may play the rôle of indirectly bringing about proteolysis of such proteins in a medium which contains, in addition to the native proteins, organic nitrogen that is readily assimilated, the following experiments were carried out.

Large test-tubes containing 10 to 20 cc. of egg albumin medium (solution of pure egg albumin and inorganic salts previously described), to which 0.5 per cent peptone had been added, were inoculated with the putrefactive anaërobes, and incubated at 37°C. for five to fourteen days. Coagulation tests were then made and the loss of coagulable protein was determined. The results of three different series of tests were as follows.

*Experiment A.* The uninoculated tube of medium (control) yielded 0.1+ gm. of coagulated protein, while two tubes which were inoculated with *B. anthracis symptomatici* contained only 0.01+ and 0.02+ gm. respectively. Aside from the loss of coagulable protein, there were other indications of putrefactive decomposition, particularly odor and discoloration of the medium. The tubes had been incubated five days.

*Experiment B.* Eleven tubes of albumin-peptone medium were employed, of which two were left uninoculated, four were inoculated with *B. anthracis symptomatici*, and five with *B. edematis maligni*. At the end of the incubation period, which was ten days, the two control tubes contained 0.0210 gm. and 0.0217 gm. of coagulable albumin, whereas in all the inoculated tubes the coagulable protein had been completely digested, thus making final filtration and weighing unnecessary.

*Experiment C.* In this last series of tests, in which the tubes were kept at 37°C. for fourteen days, eighteen different tubes were used, of which two served as uninoculated controls, eight were inoculated with *B. anthracis symptomatici*, and the same number with *B. edematis maligni*. The amounts of coagulable proteins in the controls at the end of the period were 0.0684 gm. and 0.0679 gm. No coagulum could be obtained in any of the remaining sixteen tubes.

While the putrefactive anaërobes, like the aërobes and facultative anaërobes previously studied, are unable to initiate proteoly-

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While the putrefactive anaërobes, like the aërobes and facultative anaërobes previously studied, are unable to initiate proteoly-

sis of a native protein in a medium which contains no organic or other available nitrogen aside from the protein itself, they may indirectly bring about complete decomposition with the aid of other substances, like peptone, which readily furnish the nitrogen (and carbon as well) that is necessary for early bacterial development. It must be assumed that in this early decomposition enzymes are produced in sufficient amounts to cause proteolysis or digestion of the native protein. This view is the only one that appears to be tenable in connection with the work of the so called analytic bacteria in nature. The bacterial decomposition of animal and vegetable protein matter, which is of so much economic importance, is made possible through the agency of an intermediary, like the nitrogenous products of autolysis, or any other nitrogenous substance which furnishes sufficient nitrogen for the early development of the proteolytic bacteria and for the production of the enzymes which in themselves bring about the proteolysis.

It is indeed surprising that no proteolysis should take place in a medium which contains pure native protein as the only possible source of nitrogen, even though the experiments extend over long periods of time, and at least small numbers of bacteria are constantly present. It was to be anticipated that even a slight increase in the number of bacteria, which at times took place in the early part of our individual experiments, would be accompanied by the production of proteolytic enzymes, however small the amount. The absence of proteolysis may be regarded as an indication that the enzymes which are most directly concerned in the "bacterial" decomposition of these proteins differ from ordinary pepsin and trypsin in that they have no apparent action in very high dilution, whereas the action of pepsin and trypsin does not depend on definite concentration.

#### GENERAL CONCLUSIONS.

The results of the present investigation are in full accord with the observations of Bainbridge on the behavior of certain aërobes and facultative anaërobes towards pure animal proteins. Our experiments have shown, furthermore, that the inability of bacteria to attack and decompose native proteins is not limited to these two groups of organisms, but that even the well known and

extremely active putrefactive anaërobes are devoid of this property. They have demonstrated also that pure vegetable proteins, as for example edestin, show the same resistance to the direct action of bacteria.

Solutions of native proteins may undergo complete proteolysis, however, if they contain peptone or some other nitrogenous food material which readily furnishes the necessary nitrogen for bacterial development. In such instances the proteolysis of the native protein is the immediate result of the action of an enzyme which has been elaborated by the bacteria during the process of rapid multiplication. This multiplication is made possible by the nitrogen-containing material which is present along with the native protein.

The resistance of native proteins to direct decomposition by bacteria is not due to any antiseptic properties of the proteins, but to a construction of the molecule which renders it relatively stable, the component parts being so firmly bound together that a strong cleavage-producing agent, as extreme heat, strong acids and alkalies, and enzymes, is required to change them so that bacteria may utilize their products for cell nutrition.





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CONCERNING THE ORGANIC PHOSPHORIC ACID  
COMPOUND OF WHEAT BRAN. IV.

THE OCCURRENCE OF INOSITE TRIPHOSPHATE IN WHEAT  
BRAN.

ELEVENTH PAPER ON PHYTIN.

By R. J. ANDERSON.

(From the Chemical Laboratory of the New York Agricultural Experiment  
Station, Geneva, and the First Chemical Institute of the  
University of Berlin, Berlin.)

(Received for publication, November 7, 1914.)

INTRODUCTION.

The only definitely homogeneous organic phosphoric acid ever isolated from wheat bran, as far as we are aware, is the crystalline inosite monophosphate which we described in a previous paper.<sup>1</sup> This substance differs from other inosite phosphoric acids in that it crystallizes readily, and particularly in that its barium salt is very soluble in water; *i.e.*, it is not precipitable with barium hydroxide.

Wheat bran, however, contains other organic phosphoric acids which are precipitated with barium hydroxide. It is evident from the work which we last reported on the subject<sup>2</sup> that this water-insoluble barium salt is not a homogeneous substance, but that it is a mixture of various organic phosphoric acids.

Patten and Hart<sup>3</sup> isolated an acid from this mixture of the water-insoluble barium salts, which they believed to be identical with phytic acid, or the "anhydrooxymethylene diphosphoric acid" of Posternak.

<sup>1</sup> R. J. Anderson: this *Journal*, xviii, p. 441, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 36, 1914.

<sup>2</sup> Anderson: this *Journal*, xviii, p. 425, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 36, 1914.

<sup>3</sup> A. J. Patten and E. B. Hart: *Am. Chem. Jour.*, xxxi, p. 564, 1904.

We have shown,<sup>4</sup> however, by the analyses of numerous preparations freed from inorganic phosphate, that the above substance differs entirely in composition from phytic acid or salts of this acid. Although we were able to separate this substance into various fractions which all differed in composition, it was impossible to obtain definitely homogeneous compounds by the method employed. The separation was the more difficult since neither the barium salt nor other salts with inorganic bases crystallized.

In our previous work on wheat bran we had always used the crude wheat bran ordinarily sold in the market for cattle feed. This product was not a pure bran, but contained various impurities. It is not unlikely that these impurities also contained organic phosphoric acids which would render the separation of pure compounds from the final mixture more difficult.

For the work reported in this paper we have used a perfectly pure wheat bran which was especially prepared from winter wheat for us in a local mill.

From this pure bran the organic phosphoric acids were isolated as barium salts in the usual way (compare experimental part). The acid barium salt finally obtained was shaken up with cold water in which about one-half of the total substance dissolved.

The examination of the water-insoluble portion forms the subject of this paper. We hope later on to be able to separate the water-soluble substance also into its constituents.

The portion insoluble in water, as was shown in our last report,<sup>5</sup> contains a higher percentage of phosphorus and a lower percentage of carbon than the water-soluble substance.

Since the barium salt of the above organic phosphoric acid, as well as salts with other inorganic bases, does not crystallize, we tried to prepare salts with organic bases in the hope of obtaining crystalline compounds. It was found, however, that salts with phenylhydrazine, hydrazine hydrate, aniline, pyridine, etc., showed no more tendency to crystallize than those with inorganic bases; and the brucine salt is too soluble to crystallize well.

<sup>4</sup> Anderson: this *Journal*, xii, p. 447, 1912; xviii, p. 425, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 22, 1912, and No. 36, 1914.

<sup>5</sup> Anderson: this *Journal*, xviii, p. 425, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 36, 1914.

It was found finally that strychnine gave a readily crystalline salt with the above acid. This strychnine salt separates from the hot aqueous solution on cooling in long needle-shaped crystals, or sometimes in large thin plates, depending upon the concentration. The salt was recrystallized several times from water.

The substance thus purified had practically the same composition and melting point as the salts described by Clarke,<sup>6</sup> which he had prepared from wild Indian mustard.

However, it must be noted that the molecular weight of such strychnine salts is so high that it is impossible accurately to determine the composition of the acid from the analysis of such compounds. We have used the strychnine salts, therefore, merely as a means of purification. After the salt had been several times recrystallized, the strychnine was removed and the acid again precipitated with barium hydroxide.

The substance was then analyzed; and the composition agreed very closely with a neutral barium salt of inosite triphosphate,  $C_6H_9O_{15}P_3Ba_3$ . An acid barium salt was prepared by precipitating the hydrochloric acid solution of the neutral salt with alcohol. On analysis results were obtained which agreed with a compound of the following composition:  $C_{18}H_{35}O_{45}P_9Ba_5$ , which apparently represents three molecules of inosite triphosphate joined by five atoms of barium.

The free acid was prepared from the barium salt and analyzed. The results agreed closely with the calculated composition of inosite triphosphate,  $C_6H_{15}O_{15}P_3$ .

Unfortunately the barium salts were obtained only as amorphous or granular powders, and the free acid itself was a non-crystallizable syrup. We believe, nevertheless, that the substance represents a pure compound because it had been separated previously as a crystalline strychnine salt which appeared perfectly homogeneous.

On cleavage with dilute sulphuric acid in a sealed tube at a temperature of  $150^\circ$ , inosite triphosphate decomposes into inosite and phosphoric acid.

<sup>6</sup> G. Clarke: *Jour. Chem. Soc.*, cv, p. 535, 1914.



## EXPERIMENTAL PART.

*Isolation of the substance from wheat bran as a barium salt.*

The wheat bran was digested over night in 0.2 per cent hydrochloric acid. It was then strained through cheese cloth and filtered. The free acid in the extract was nearly neutralized by adding a dilute solution of barium hydroxide until a slight permanent precipitate remained. A concentrated solution of barium chloride was then added and the precipitate allowed to settle over night. The supernatant liquid was syphoned off and the residue centrifugalized. The precipitate was finally brought upon a Büchner funnel, freed as much as possible from the mother-liquor, and then washed with 30 per cent alcohol. For further purification the substance was precipitated alternately six times from about 1 per cent hydrochloric acid with barium hydroxide (Kahlbaum, alkali-free), and six times from the same strength hydrochloric acid with alcohol.

After this treatment the substance was obtained as a snow-white amorphous powder. It was free from inorganic phosphate, and bases other than barium could not be detected. But it still contained oxalates from which it was freed in the manner described in a former paper.<sup>7</sup> The substance was finally precipitated from about 1 per cent hydrochloric acid with alcohol, filtered, and washed free of chlorides with dilute alcohol. It was finally washed with alcohol and ether and dried in vacuum over sulphuric acid. The dry substance weighed about 66 grams.

This crude acid salt was rubbed up in a mortar with about 500 cc. of cold water and allowed to stand a few hours. It was then filtered and washed successively with water, alcohol, and ether, and was dried in vacuum over sulphuric acid. The water-insoluble residue weighed about 30 grams.

The water-soluble portion in the filtrate was precipitated with barium hydroxide and reserved for a future investigation.

<sup>7</sup> Anderson: this *Journal*, xviii, p. 425, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 36, 1914.

*Preparation of the strychnine salt.*

The water-insoluble portion of the barium salt mentioned above was suspended in water and the barium precipitated with slight excess of dilute sulphuric acid. The barium sulphate was removed and the filtrate precipitated with excess of copper acetate. The copper precipitate was filtered and washed with water until the washings gave no reaction with barium chloride. It was then suspended in water, and the copper was precipitated with hydrogen sulphide and filtered off.

The solution containing the free organic phosphoric acid was diluted to about 2 liters with water, heated on the water bath, and 44 grams of powdered strychnine were added. In a few minutes the strychnine was dissolved. The solution was filtered and concentrated in vacuum at a temperature of 45°–50° to about one-half the volume. The strychnine salt soon began to separate in long needle-shaped crystals. After standing in the ice chest over night, the crystals were filtered off, washed in ice cold water followed by absolute alcohol and ether, and dried in the air. Yield, 45.8 grams.

For further purification the substance was twice recrystallized from hot water. It was then obtained in pure white needle-shaped crystals which looked perfectly homogeneous.

From more concentrated solutions the substance sometimes separates in the form of colorless plates which differ from the needle-shaped crystals in that they contain about 4 per cent more of water of crystallization.

The strychnine salt has no sharp or definite melting point. Heated in a capillary tube it softens at about 200°C., but it does not melt completely even at a much higher temperature. On moist litmus paper it shows an acid reaction. The substance does not change in color on drying at 105°C.

After drying at 105°C. in vacuum over phosphorus pentoxide, it was analyzed. For combustion the substance was mixed with fine copper oxide.

The needle-shaped crystals gave the following:

0.1534 gram substance gave 0.0901 gram  $\text{H}_2\text{O}$  and 0.3329 gram  $\text{CO}_2$ .

0.2944 gram substance gave 0.0702 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.1175 gram substance gave 6.75 cc. N at 22°C. and 763 mm.

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0.1688 gram substance lost 0.0154 gram  $H_2O$  on drying.  
0.1982 gram substance lost 0.0183 gram  $H_2O$  on drying.  
Found: C=59.18; H=6.57; P=6.64; N=6.56;  $H_2O$ =9.12 and 9.23 per cent.

The plate-shaped crystals gave the following:

0.1786 gram substance gave 0.0993 gram  $H_2O$  and 0.3909 gram  $CO_2$ .  
0.4972 gram substance gave 0.1089 gram  $Mg_2P_2O_7$ .  
0.1955 gram substance lost 0.0256 gram  $H_2O$  on drying.  
0.5997 gram substance lost 0.0783 gram  $H_2O$  on drying.  
Found: C=59.60; H=6.22; P=6.10;  $H_2O$ =13.09 and 13.05 per cent.

These compounds apparently do not represent definite strychnine salts of inosite triphosphate; but they seem to be mixtures of the tri- and tetrastrychnine salts.

For tristrychnine inosite triphosphate,  $C_6H_{18}O_{18}P_3(C_{21}H_{22}N_2O_2)_3=1422$ .  
Calculated: C=58.23; H=5.70; P=6.54; N=5.90 per cent.  
For tetrastrychnine inosite triphosphate,  $C_6H_{18}O_{18}P_3(C_{21}H_{22}N_2O_2)_4=1756$ .  
Calculated: C=61.50; H=5.86; P=5.30; N=6.37 per cent.

### *Preparation of the barium salt from the strychnine salt.*

The recrystallized strychnine salt, 27 grams, was dissolved in about 750 cc. of hot water, and the solution rendered alkaline with ammonia. After standing in ice water for some time the strychnine was filtered off. The filtrate was shaken with several portions of chloroform to remove the last traces of strychnine.

The solution, which now contained the ammonium salt of the organic phosphoric acid, was precipitated by adding a solution of barium chloride in excess. After settling over night, the precipitate was filtered and washed several times with water. It was then dissolved in 1 per cent hydrochloric acid, filtered, and precipitated with barium hydroxide in excess. The precipitate was filtered and washed with water until free from chlorides. It was again dissolved in 1 per cent hydrochloric acid, filtered, and the solution rendered neutral to litmus with barium hydroxide. The precipitate was filtered and washed free of chlorides with water and then in alcohol and ether and dried in vacuum over sulphuric acid. The substance was a pure white amorphous powder. On moist litmus paper it showed a very faint acid reaction. It was free from nitrogen.

For analysis it was dried at 100° in vacuum over phosphorus pentoxide.

0.2323 gram substance gave 0.0370 gram  $H_2O$  and 0.0758 gram  $CO_2$ .  
0.2085 gram substance gave 0.1668 gram  $BaSO_4$  and 0.0868 gram  $Mg_2P_2O_7$ .  
Found: C=8.89; H=1.78; P=11.60; Ba=47.07 per cent.

The substance was again dissolved in 1 per cent hydrochloric acid and the solution neutralized to litmus with barium hydroxide. The precipitated substance was filtered, washed free of chlorides with water, then washed with alcohol and ether, and dried in vacuum over sulphuric acid.

It was analyzed after drying as before, and the following results were obtained:

0.3168 gram substance gave 0.0481 gram  $H_2O$  and 0.0972 gram  $CO_2$ .  
0.2366 gram substance gave 0.1968 gram  $BaSO_4$  and 0.0960 gram  $Mg_2P_2O_7$ .  
Found: C=8.36; H=1.69; P=11.31; Ba=48.94 per cent.  
For the neutral barium salt of inosite triphosphate,  $C_6H_9O_{11}P_3Ba_3=826$ .  
Calculated: C=8.71; H=1.08; P=11.25; Ba=49.88 per cent.

In the two analyses reported above the carbon is somewhat low. It must be noted, however, that these barium salts burned with extreme difficulty. Traces of carbon remained after prolonged heating in a current of oxygen. When the residues were mixed with fine copper oxide and reburned, 1 or 2 mgm. more of carbon dioxide were obtained; but we believe that the combustion, even under these conditions, was incomplete.

#### *Preparation of the acid barium salt.*

The above neutral barium salt was dissolved in the minimum quantity of 1 per cent hydrochloric acid; the solution was filtered and precipitated by adding about an equal volume of alcohol. The resulting precipitate was filtered, washed free of chlorides with dilute alcohol, washed further with absolute alcohol and ether, and dried in vacuum over sulphuric acid. The substance was then a pure white amorphous powder which showed a strong acid reaction on moist litmus paper. It was analyzed after drying at 105°C. in vacuum over phosphorus pentoxide.

0.2588 gram substance gave 0.0538 gram  $H_2O$  and 0.1046 gram  $CO_2$ .  
0.1691 gram substance gave 0.1027 gram  $BaSO_4$  and 0.0890  $Mg_2P_2O_7$ .  
Found: C=11.02; H=2.32; P=14.67; Ba=35.74 per cent.

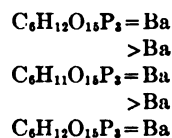
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In the combustion of this substance a practically white ash was obtained.

This compound is evidently a complex acid salt of inosite triphosphate and agrees with the following formula:



(calculated: C = 11.15; H = 1.80; P = 14.40; Ba = 35.46 per cent), which may be graphically represented as follows:



That is, three acid molecules of inosite triphosphate joined by five atoms of barium. Whether it is a compound as represented above or a mixture of various acid salts of inosite triphosphate can hardly be determined.

### *Preparation of the free acid.*

The barium salt from above was suspended in water and the barium precipitated with a slight excess of dilute sulphuric acid. The barium sulphate was filtered off and the filtrate precipitated with excess of copper acetate. The copper precipitate was filtered and washed with water until it gave no reaction with barium chloride. It was then suspended in water and decomposed with hydrogen sulphide. The copper sulphide was filtered off, the filtrate evaporated in vacuum at a temperature of 40°–45°C. to small bulk, and the residue dried in vacuum over sulphuric acid. The substance was then obtained as a practically colorless syrup. After continued drying it forms a hard, sticky, hygroscopic mass. It is extremely soluble in water, and also readily soluble in dilute and absolute alcohol. Much time was consumed in endeavoring to obtain it in crystalline form, but without success. The syrupy substance was therefore analyzed after drying: first, for several days in vacuum over sulphuric acid at room temperature; and finally at 100°C. in vacuum over phosphorus pentoxide. On drying at this temperature the color turned quite dark.

0.1685 gram substance gave 0.0557 gram  $\text{H}_2\text{O}$  and 0.1054 gram  $\text{CO}_2$ .  
0.1693 gram substance gave 0.1363 gram  $\text{Mg}_3\text{P}_2\text{O}_7$ .  
Found: C=17.06; H=3.69; P=22.44 per cent.  
For inosite triphosphate,  $\text{C}_6\text{H}_{18}\text{O}_{18}\text{P}_3=420$ .  
Calculated: C=17.14; H=3.57; P=22.14 per cent.

In the above combustion a slight residue of unburned carbon remained enclosed in the fused metaphosphoric acid. It was mixed with some fine copper oxide and again burned, when a few additional milligrams of carbon dioxide were obtained.

*Properties of inosite triphosphate.*

The reactions of this acid differ in several particulars from phytic acid or inosite hexaphosphate.

The concentrated aqueous solution gives no precipitate with ammonium molybdate either in the cold or on warming. The cold aqueous solution of inosite hexaphosphate gives a white crystalline precipitate with ammonium molybdate.

The aqueous solution of the acid is not precipitated with silver nitrate, while inosite hexaphosphate gives a white amorphous precipitate. However, a solution of inosite triphosphate neutralized with ammonia gives a white amorphous precipitate with silver nitrate.

An aqueous solution of inosite triphosphate when added to a solution of egg albumin causes only a slight turbidity. On longer standing a white precipitate separates slowly. Inosite hexaphosphate precipitates egg albumin immediately.

The acid is very soluble in water, and it is readily soluble in both dilute and absolute alcohol. From the latter solution it is precipitated by ether in small oily drops.

The acid is not precipitated by barium or calcium chlorides; but alcohol added to the water solutions of acid and chlorides produces white amorphous precipitates. The calcium and barium salts are likewise precipitated with ammonia.

*Cleavage of inosite triphosphate into inosite and phosphoric acid.*

One gram of the acid, dissolved in a little water, was heated with 10 cc. of  $\frac{5}{N}$  sulphuric acid in a sealed tube for three hours at  $150^\circ$ – $155^\circ$ . The contents of the tube were then slightly yellowish

brown in color. The sulphuric and phosphoric acids were precipitated with barium hydroxide and the inosite isolated in the usual way. Unfortunately a portion of the solution was lost, but from what remained 0.15 of a gram of inosite was obtained. After twice recrystallizing from dilute alcohol with the addition of ether 0.12 of a gram of inosite in the characteristic needle-shaped crystals was obtained. It gave the reaction of Scherer and melted at 222°C. (uncorrected). After mixing with some previously isolated and analyzed inosite, the melting point did not change. The substance was therefore undoubtedly inosite, and the analysis was omitted.

Although the barium salts described in this paper were amorphous and the inosite triphosphate itself was a non-crystallizable syrup, we believe that the substances were pure. The basis for belief rests upon the fact that they had been prepared from the recrystallized strychnine salt, which appeared perfectly homogeneous.

We have been unable to complete the investigation of the water-soluble portion of the barium salt mentioned on page 466. We wish, however, to record the following data, although they are very incomplete.

After the aqueous solution had been precipitated with barium hydroxide, as already mentioned, the precipitate was filtered and washed in water. It was then dissolved in the minimum quantity of about 2 per cent hydrochloric acid, filtered, and precipitated with about an equal volume of alcohol. The voluminous amorphous precipitate was filtered and washed free of chlorides with dilute alcohol, washed with absolute alcohol and ether, and dried in vacuum over sulphuric acid. The dry substance weighed 38 grams.

It was again rubbed up in a mortar with about 200 cc. of cold water. After it had stood a short while, it was filtered, washed with water, alcohol, and ether, and dried in vacuum over sulphuric acid. The water-insoluble portion weighed 10.3 grams. The filtrate was concentrated in vacuum at a temperature of 40° to about 100 cc. As the concentration proceeded, a small quantity of a heavy white barium salt separated. It was not definitely crystalline and it weighed only 0.9 of a gram. The aqueous solution, about 100 cc., contained, therefore, about 27 grams of the

original substance. It was found impossible to obtain anything crystalline from this solution. However, on heating to boiling, a heavy, semicrystalline or granular powder separated. This was filtered and washed in hot water, alcohol, and ether, and dried in the air. It weighed 1.7 grams.

The filtrate from the above was precipitated by adding about half a volume of alcohol. The amorphous white precipitate was filtered, washed, and dried in vacuum over sulphuric acid.

A further quantity of substance was precipitated by adding more alcohol to the filtrate.

These three fractions were analyzed after drying at 105° in vacuum over phosphorus pentoxide.

The semicrystalline or granular powder gave the following:

C=10.14; H=1.90; P=13.18; Ba=39.60; H<sub>2</sub>O=12.11 per cent.

These results agree closely with the figures for a dibarium salt of inosite triphosphate.

For  $C_6H_{11}O_{13}P_3Ba_2$ .

Calculated: C=10.41; H=1.59; P=13.45; Ba=39.79 per cent.

The first amorphous precipitate gave:

C=12.07; H=2.30; P=13.93; Ba=33.70 per cent.

The second amorphous precipitate gave:

C=13.54; H=2.74; P=13.22; Ba=32.38 per cent.

Lack of time has prevented the further examination of these substances, but we hope to complete the investigation later.

From the results recorded it is evident that the organic phosphoric acid contained in the preparation used in this investigation consisted principally of inosite triphosphate,  $C_6H_{13}O_{13}P_3$ . The amorphous barium salts analyzed above may have contained some inosite diphosphate or some other organic phosphoric acid. In addition to the above it must be noted that inosite monophosphate, described in an earlier paper, had been isolated from this same wheat bran.

The author desires to express his appreciation and thanks to His Excellency, Prof. E. Fischer, for the interest shown in the work reported in this paper.





# THE HYDROLYSIS OF PHYTIN BY THE ENZYME PHYTASE CONTAINED IN WHEAT BRAN.

TWELFTH PAPER ON PHYTIN.

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## INTRODUCTION.

It has been shown by the investigations of Suzuki, Yoshimura, and Takaishi<sup>1</sup> that rice bran contains an enzyme which rapidly hydrolyses phytin with formation of inosite and inorganic phosphoric acid. These authors concluded that wheat bran likewise contained a similar enzyme, because the inorganic phosphorus increased in wheat bran extracts on standing.

Plimmer<sup>2</sup> examined a large number of extracts prepared from the intestines, liver, pancreas, castor beans, etc., as to their action on organic phosphorus compounds. While some of these showed a slight cleavage action on phytin, none could be compared in activity to an aqueous extract of wheat bran. The hydrolytic action of these extracts was determined by estimating from time to time the amount of inorganic phosphorus split off from phytin solutions of known concentration.

Since the above experiments clearly demonstrated that large quantities of inorganic phosphate were liberated from phytin by wheat bran extracts, it appeared of interest to determine what products, in addition to inosite and inorganic phosphoric acid, were formed under these conditions. For this purpose wheat bran extract was allowed to act upon a dilute solution of phytin

<sup>1</sup> U. Suzuki, K. Yoshimura, and M. Takaishi: *Bulletins of the College of Agriculture*, Tokyo, vii, pp. 503-512, 1907.

<sup>2</sup> R. H. A. Plimmer: *Biochem. Jour.*, vii, p. 43, 1913.

at a temperature of 37°. Inorganic phosphoric acid was determined in the solution from time to time.

It was found that about two-thirds of the total phosphorus was split off during the first sixteen days. Afterwards there was no appreciable change even on standing for about two years.

The solution had been prepared and the original determinations made by Dr. Plimmer. At his suggestion the writer undertook to examine the final reaction mixture for such products as had been formed.

These products were separated into two portions by precipitating the original solution with barium hydroxide. The precipitate contained inorganic barium phosphate and also those barium salts of organic phosphoric acids that were insoluble in the dilute alkaline solution. The filtrate, on the other hand, was found to contain inosite monophosphate, and free inosite.

The inorganic phosphate and other impurities were removed from the crude barium hydroxide precipitate, as will be described in the experimental part. The organic phosphoric acids which remained were obtained as amorphous barium salts. It was impossible to isolate any unchanged barium phytate. It is evident then that all the phytin had been partially hydrolyzed.

The above amorphous substance appeared to consist mainly of barium inosite triphosphate, but probably mixed with some barium inosite diphosphate. Owing to the difficulty of separating these compounds their isolation was not attempted.

Among the soluble substances which had been formed we were able to isolate and identify inosite monophosphate, a substance which we have previously isolated from wheat bran.<sup>3</sup> In addition to this, the solution also contained some free inosite which was isolated by means of its lead compound.

The action of this enzyme, phytase, upon phytin appears to proceed in several stages. Only a portion of the phytin is completely decomposed into inosite and phosphoric acid, but all the phytin is partially hydrolyzed with formation of certain lower phosphoric acid esters of inosite; *viz.*, inosite tri-, di-, and monophosphate, and inorganic phosphoric acid. The formation of these intermediate products is only possible through the destruc-

<sup>3</sup> R. J. Anderson: this *Journal*, xviii, p. 441, 1914; and *New York Agricultural Experiment Station Technical Bulletins*, No. 36, 1914.

tion or inhibition of the enzyme before the hydrolysis is complete. The reason for this inhibition is not clear, but it may be due to the excess of phosphoric acid which is liberated.

It is interesting to note, and we call particular attention to the fact, that the organic phosphoric acids which remain as intermediate products of the action of the enzyme upon phytin, *viz.*, inosite triphosphate and inosite monophosphate, are identical with the substances which we have isolated previously from wheat bran after it has been digested in 0.2 per cent hydrochloric acid.

#### EXPERIMENTAL PART.

Commercial phytin, 100 grams, was dissolved in 500 cc. of water and filtered from the insoluble matter which weighed 3.5 grams when dried at 100°. The pale yellow solution was treated with 38 grams of oxalic acid dissolved in about 250 cc. of water. The calcium oxalate was filtered off, washed, and dried. It weighed 48 grams. The solution was diluted to 6000 cc. with water and was then found to contain 40 grams of phosphorus pentoxide. To it were added 800 cc. of an aqueous extract of wheat bran which contained 2.2 grams of  $P_2O_5$ . The solution was kept under toluene at a temperature of 37°. No hydrolysis occurred in a week. This was evidently due to the strongly acid reaction of the solution. It was nearly neutralized with ammonia, and 735 cc. of bran extract containing 1.53 grams of  $P_2O_5$  were added. It was again kept at a temperature of 37° under toluene. In nine days one-half of the total phosphorus was hydrolyzed; in sixteen days two-thirds was hydrolyzed. In thirty-five days the amount of hydrolysis had not altered, and after about two years it was again the same. The total and inorganic phosphorus was determined as described by Plimmer and Page.<sup>4</sup>

The dark colored solution was filtered and barium hydroxide (Kahlbaum) added in slight excess. After standing over night the precipitate was filtered and washed in water. The filtrate and washings were evaporated on the water bath and the residue was examined, as will be described later.

The barium precipitate was dissolved in about 2.5 per cent hydrochloric acid, filtered, and precipitated by adding about an

<sup>4</sup> R. H. A. Plimmer and H. J. Page: *Biochem. Jour.*, vii, p. 162, 1913.

equal volume of alcohol. The precipitate was filtered and washed in dilute alcohol. The substance was again precipitated four times in the same way. It was then precipitated by barium hydroxide three times from about 2 per cent hydrochloric acid, and finally twice more with alcohol from the same strength hydrochloric acid. After finally filtering it was washed free of chlorides in dilute alcohol and then in alcohol and ether and dried in vacuum over sulphuric acid. The substance was then a snow-white amorphous powder. It weighed 28.4 grams. It was free from chlorides and inorganic phosphate, and bases other than barium could not be detected.

The substance was then rubbed up in a mortar with 300 cc. of cold water and allowed to stand with occasional shaking for a few hours. It was then filtered and washed in water, alcohol, and ether, and dried in vacuum over sulphuric acid. The dry water-insoluble portion weighed 5.4 grams.

The filtrate from above was neutralized to litmus with barium hydroxide. The precipitate was filtered and washed in water, alcohol, and ether, and dried in vacuum over sulphuric acid. It weighed 23.6 grams.

These precipitates were analyzed after drying at 105° in vacuum over phosphorus pentoxide.

The first, water-insoluble portion gave the following result:

Found: C = 11.46; H = 1.93; P = 11.59; Ba = 39.94 per cent.

This substance is apparently largely composed of the dibarium inosite triphosphate.

Calculated for this:  $C_6H_{11}O_{15}P_3Ba_2 = 690$ .

C = 10.43; H = 1.59; P = 13.47; Ba = 39.71 per cent.

It is, however, not pure, but apparently contains some barium inosite diphosphate; because the carbon is high and the phosphorus is low.

The water-soluble substance which was precipitated with barium hydroxide gave the following:

Found: C = 9.63; H = 1.63; P = 10.91; Ba = 47.41 per cent.

This substance also appears to consist largely of the neutral barium salt of inosite triphosphate.

Calculated for the latter:  $C_6H_9O_{15}P_3Ba_4 = 826$ .

C = 8.71; H = 1.08; P = 11.25; Ba = 49.88 per cent.

The carbon, however, is high, and the phosphorus as well as the barium are low; which points to the presence of barium inosite diphosphate.

In the hope of approximately separating these barium inosite tri- and diphosphates, the substance, 23.6 grams, was digested in dilute acetic acid for several hours with occasional shaking. It was then filtered and washed in water, and the insoluble portion dried in vacuum over sulphuric acid. It weighed 10 grams.

The filtrate and washings containing the soluble portion of the substance were precipitated by adding lead acetate in excess. After standing over night the white amorphous precipitate was filtered and washed in water. It was suspended in water and decomposed by hydrogen sulphide, filtered, and the excess of hydrogen sulphide boiled off. It was again precipitated in the same manner with lead acetate and decomposed with hydrogen sulphide. The solution still contained a considerable quantity of barium. The barium was therefore removed with a slight excess of dilute sulphuric acid. After filtration of the barium sulphate the solution was precipitated by adding copper acetate in excess. The copper precipitate was filtered, washed, suspended in water, and decomposed with hydrogen sulphide. After removing the copper sulphide, the filtrate was evaporated in vacuum to small bulk, and finally dried in vacuum over sulphuric acid. There remained a thick, nearly colorless syrup. It was readily soluble in alcohol. The addition of chloroform to this solution caused the substance to separate in small oily drops; the addition of ether produced a cloudiness, and on standing a flocculent amorphous precipitate separated. These solutions could not be brought to crystallize. The acid preparation itself was kept for several weeks in the desiccator over sulphuric acid. It became a hard, sticky mass, but showed absolutely no tendency to crystallize. The color of the preparation, kept in this manner, gradually darkened.

Since the acid would not crystallize, the syrupy substance was analyzed after drying at  $105^\circ$  in vacuum over phosphorus pentoxide.

Found: C = 18.58; H = 3.82; P = 20.38 per cent.

Calculated for inosite triphosphoric acid:  $C_6H_{11}O_{11}P_3 = 420$ .

C = 17.14; H = 3.57; P = 22.14 per cent.

Calculated for inosite diphosphoric acid:  $C_6H_{11}O_{10}P_2 = 340$ .

C = 21.17; H = 4.11; P = 18.23 per cent.

This acid preparation is evidently also a mixture of the inosite tri- and diphosphoric acids.

EXAMINATION OF THE FILTRATE AFTER THE WATER-INSOLUBLE  
BARIUM SALTS HAD BEEN PRECIPITATED.

The filtrate was evaporated, as mentioned on page 477, and the residue taken up in hot water. It was decolorized with animal charcoal. The solution was neutral in reaction. It strongly reduced Fehling's solution on boiling, possibly due to sugars introduced with the bran extract. The solution was found to contain barium and also phosphorus in organic combination, evidently inosite monophosphate. The aqueous solution was precipitated by adding about an equal volume of alcohol and the white amorphous precipitate filtered off, the filtrate being reserved for further examination.

*Isolation of inosite monophosphate.* The above precipitate, which formed on the addition of alcohol, was dissolved in water, slightly acidified with acetic acid, and then precipitated with lead acetate in excess. After settling, this was filtered, washed in water, suspended in hot water, and decomposed with hydrogen sulphide. It was then filtered, and the filtrate boiled to expel excess of hydrogen sulphide. It was reprecipitated several times with lead acetate in the same manner until a white lead precipitate was obtained. This was finally decomposed with hydrogen sulphide, filtered, and evaporated to small bulk in vacuum and then dried in vacuum over sulphuric acid until a thick syrup remained. On scratching with a glass rod, this crystallized to a white solid mass. It was digested in alcohol and filtered, washed in alcohol and ether, and dried in the air. It weighed 1.6 grams. It had all the properties of inosite monophosphate. For further purification it was dissolved in a few cubic centimeters of water and filtered. Alcohol was then added until the solution turned cloudy; it was heated

until it cleared up, and more alcohol was added until a faint permanent cloudiness remained. It was allowed to stand for about forty-eight hours at room temperature, when the substance had separated in massive, practically colorless crystals. After filtering, washing in alcohol and ether, and drying in the air, 1 gram of substance was obtained. When heated in a capillary tube it began to soften at  $188^{\circ}$ – $189^{\circ}$  and melted with decomposition and effervescence at  $190^{\circ}\text{C}$ . (uncorrected). The appearance and properties of the substance corresponded exactly with those described for inosite monophosphate and the analysis was therefore omitted.

*Isolation of inosite.* The filtrate, after precipitation of the above barium salt of inosite monophosphate with alcohol, was evaporated on the water bath until the alcohol was removed. It still contained barium, chlorides, etc. The barium was quantitatively precipitated with dilute sulphuric acid and the solution again concentrated on the water bath.

The addition of lead acetate caused no precipitate. Basic lead acetate was then added as long as any precipitate formed. This precipitate was filtered off and discarded. The solution was then heated to boiling, more basic lead acetate added, and the solution was finally made strongly alkaline with ammonia and allowed to stand over night. This precipitate was filtered and washed in water, and then decomposed in aqueous suspension with hydrogen sulphide. The filtrate was concentrated on the water bath and the inosite brought to crystallization by the addition of alcohol. After recrystallizing several times 0.5 of a gram of pure inosite was obtained in the characteristic needle-shaped crystals. It gave the reaction of Scherer and melted at  $218^{\circ}\text{C}$ . (uncorrected).

#### SUMMARY.

The chief products of the hydrolysis of phytin by the phytase in wheat bran are inorganic phosphoric acid and certain intermediate compounds apparently consisting of inosite tri-, di-, and monophosphates. These intermediate substances are identical with the compounds which we have previously isolated from 0.2 per cent hydrochloric acid extracts of wheat bran.



A portion of the phytin was completely hydrolyzed by the action of the enzyme into phosphoric acid and inosite; because the solution was found to contain some free inosite.

All the phytin was at least partially hydrolyzed; since the final reaction mixture did not contain any unchanged inosite hexaphosphate.

# THE HYDROLYSIS OF THE ORGANIC PHOSPHORUS COMPOUND OF WHEAT BRAN BY THE ENZYME PHYTASE.

THIRTEENTH PAPER ON PHYTIN.

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(Received for publication, January 9, 1915.)

## INTRODUCTION.

In several previous papers<sup>1</sup> on the subject of the organic phosphoric acid compounds of wheat bran, we have mentioned that when wheat bran is digested in 0.2 per cent hydrochloric acid, the resulting extract always contains relatively large quantities of inorganic phosphate. Up to the present we have had no data concerning the origin of this inorganic phosphate, and it remained to determine whether it was originally present in the bran or had been formed during the digestion by hydrolysis from the organic phosphorus compound.

Hart and Andrews,<sup>2</sup> who examined wheat bran, as well as a large number of other feeding materials, came to the conclusion that the phosphorus was present almost entirely in organic combination. Some criticism, however, has been offered of their method of estimating inorganic phosphorus, and it has been suggested that the time, fifteen minutes, which they allowed for digestion was not sufficient for complete extraction.<sup>3</sup>

<sup>1</sup> R. J. Anderson: this *Journal*, xii, p. 447, 1912; xviii, p. 425, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 22, 1912 and No. 36, 1914.

<sup>2</sup> E. B. Hart and W. H. Andrews: *New York Agricultural Experiment Station Bulletins*, No. 238, 1903.

<sup>3</sup> Forbes: *Ohio Agricultural Experiment Station Bulletins*, No. 215.

## 484 Organic Phosphorus Compound of Wheat Bran

From the work of Suzuki, Yoshimura, and Takaishi<sup>4</sup> and of Plimmer<sup>5</sup> it is evident that wheat bran contains an enzyme which rapidly hydrolyzes a portion of the organic phosphorus into inorganic phosphoric acid. The quantitative determinations of the activity of this enzyme on phytin or inositol hexaphosphate reported in the preceding paper show that about two-thirds of the total phosphorus of the phytin was transformed into inorganic phosphoric acid in about sixteen days. Since a small quantity of wheat bran extract is capable of hydrolyzing phytin to such an extent, it is evident that the organic phosphoric acid compound originally present in the bran would also be hydrolyzed on digestion under the same conditions.

The determinations herein reported were undertaken in the hope of throwing some light upon this question. It was also hoped to determine the cause of the large percentage of inorganic phosphoric acid in extracts obtained on digesting wheat bran in 0.2 per cent hydrochloric acid.

The results show that the enzyme phytase contained in wheat bran very rapidly hydrolyzes the organic phosphorus compound of the bran into inorganic phosphoric acid under certain conditions. We have endeavored to determine under what conditions the maximum activity of the enzyme manifests itself, and also the conditions under which its action is either inhibited or destroyed.

When wheat bran is digested in distilled water the hydrolysis begins at once and proceeds with considerable rapidity, and at the end of twenty-four hours nearly 90 per cent of the total water-soluble phosphorus is inorganic. The organic and inorganic phosphates of the bran are only partially soluble in the water. When the extracts, after digestion of the bran for twenty-four hours in water, are acidified with hydrochloric acid, only about 60 per cent of the total dissolved phosphorus compounds are inorganic (see Table II). This result is not perceptibly altered by digesting the bran in water for forty-eight hours and then acidifying (see Table III).

<sup>4</sup> U. Suzuki, K. Yoshimura, and M. Takaishi: *Bulletins of the College of Agriculture*, Tokyo, vii, pp. 503-512, 1907.

<sup>5</sup> R. H. A. Plimmer: *Biochem. Jour.*, vii, p. 43, 1913.

When the bran is digested in 0.2 per cent hydrochloric acid, the rate of hydrolysis is greater than with pure water. The actual amounts of both organic and inorganic phosphorus are greater in the dilute acid than in the aqueous extracts. After three hours about one-third of the total phosphorus is inorganic, and after twenty-four hours about three-quarters of the total soluble phosphorus has been hydrolyzed to inorganic phosphoric acid, as is shown in Table V.

With 0.2 per cent acetic acid the hydrolysis is more rapid and more complete than with 0.2 per cent hydrochloric acid, 85 per cent in twenty-four hours (Table VII); and the most complete hydrolysis was obtained with 0.1 per cent hydrochloric acid; namely, 92 per cent (Table VIII).

That the hydrolysis of the organic phosphorus compound is due to enzyme action and not to the acid employed is strikingly illustrated by the fact that with increasing strengths of the hydrochloric acid the amount of inorganic phosphorus rapidly and steadily diminishes. But when 0.5, 1.0, or 2.0 per cent hydrochloric acid is used, the inorganic phosphorus is practically constant (Table V).

The enzyme is remarkably sensitive to certain concentrations of hydrochloric acid. The hydrolyzing action is stimulated by the presence of 0.1 or 0.2 per cent hydrochloric acid, in which the action is much more rapid than in pure water; but 0.3 per cent almost inhibits the enzyme activity. With the 0.1 per cent acid 92 per cent, and with the 0.2 per cent acid 76 per cent, of the total phosphorus becomes inorganic, but with 0.3 per cent acid only about 20 per cent of the total phosphorus becomes inorganic (Tables V and VIII).

Since such a slight difference in strength of the hydrochloric acid caused such a great difference in the amount of the inorganic phosphorus, it appeared of interest to determine whether the acid merely inhibited the hydrolysis, or if the enzyme was destroyed by it. Some of the bran was digested in 0.5 per cent hydrochloric acid for one and one-half hours, and the acid then nearly neutralized with ammonia, and the whole allowed to digest for twenty-four hours. In this case there was no increase in the inorganic phosphorus, showing that hydrochloric acid of this strength completely destroys the enzyme (Table X).

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The enzyme is also destroyed by digesting the bran in 0.25 per cent ammonia, as well as by pouring boiling water or boiling 0.2 per cent hydrochloric acid over the bran (Tables X and IX).

Concerning the amount of inorganic phosphorus in wheat bran, it is evident, judging by the results obtained with 0.5, 1.0, and 2.0 per cent hydrochloric acid, as well as on treating bran with boiling water or boiling 0.2 per cent hydrochloric acid and with 0.25 per cent ammonia, that ordinary bran contains about 0.1 per cent of phosphorus as inorganic, which represents about 11 per cent of the total soluble phosphorus. It seems quite certain that under these conditions of extraction no hydrolysis of the organic phosphorus occurs, and we believe that these figures represent the amount of inorganic phosphorus normally present in wheat bran.

These results are not at variance with the findings of Hart and Andrews.<sup>6</sup> After digesting bran in 1.0 per cent hydrochloric acid for forty hours, they found 0.087 per cent of inorganic phosphorus in the extract. This is practically the same as our result, 0.081 per cent inorganic phosphorus, after digesting bran for five hours in 1.0 per cent hydrochloric acid. The same authors found only 0.036 per cent inorganic phosphorus in wheat bran after digesting in 0.2 per cent hydrochloric acid for fifteen minutes. The lower percentage is no doubt due to incomplete extraction, and we believe that the higher figures represent the actual amount present.

### EXPERIMENTAL PART.

Total and inorganic phosphorus was determined in extracts prepared from wheat bran. The extracts were prepared by digesting wheat bran in water or dilute acid for varying lengths of time, the particulars and details being given at the beginning of each table. Total phosphorus was determined after decomposing by the Neumann method. Inorganic phosphorus was determined as follows: The extract was diluted with about 50 cc. of water, 15 grams of ammonium nitrate were added, and then warmed to 65° on the water bath. It was then strongly acidified with nitric acid, the phosphorus precipitated with ammonium molybdate, and the solution kept at the above temperature for half an hour.

<sup>6</sup> Hart and Andrews: *loc. cit.*

Under these conditions there is no danger of cleavage of the organic phosphorus,—at least weighable quantities of phosphorus are not precipitated during this time from preparations which are free from inorganic phosphate. Plimmer<sup>7</sup> has also shown that it is necessary to heat phytin solutions with  $\frac{1}{N}$  or  $\frac{2}{N}$  nitric acid for several hours to a temperature of 75° or higher before appreciable quantities of inorganic phosphorus are split off.

The phosphomolybdate precipitate was then filtered off and the phosphorus determined as magnesium pyrophosphate in the usual way.

The digestions were made throughout these experiments at room temperature, about 16°C. Ten grams of the bran were digested in 100 cc. of water for the time mentioned, then filtered, and 20 cc. used for each determination. The same quantities were used throughout.

TABLE I.

TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
$\frac{1}{2}$	0.362	0.165	45.77
1	0.420	0.239	56.95
4	0.508	0.383	75.34
20	0.655	0.576	88.00
24	0.747	0.660	88.43

It will be noticed that the hydrolysis is greatest during the first four hours. The percentage of phosphorus represents only that amount which was soluble in the water.

After digesting in water for twenty-four hours and then acidifying with hydrochloric acid and shaking for half an hour the following results were obtained:

TABLE II.

TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
24	1.06	0.649	60.85

<sup>7</sup> Plimmer: *loc. cit.*, p. 72.

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After digesting the bran for forty-eight hours in water and then adding 100 cc. of 2 per cent hydrochloric acid and allowing it to stand for another twenty-four hours, the following results were obtained:

TABLE III.

TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1.27	0.803	62.79
1.28	0.796	62.13

The addition of toluene to the water appears to diminish the amount of phosphorus dissolved without affecting the degree of hydrolysis.

TABLE IV.

TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
24 with toluene	0.518	0.462	89.25

With hydrochloric acid as the extracting medium, the strength varying from 0.2 to 2.0 per cent, the following results were obtained: 10 grams of bran were digested in 100 cc. of the acid for the length of time mentioned in the table. It was then filtered and 20 cc. of the filtrate were used for each determination.

TABLE V.

SOLVENT	TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.2% HCl	3	1.130	0.415	36.52
0.2% HCl	24	1.200	0.925	76.87
0.3% HCl	24	0.999	0.203	20.22
0.4% HCl	24	0.939	0.146	15.61
0.5% HCl	20	0.922	0.124	13.44
1.0% HCl	5	0.894	0.081	9.13
2.0% HCl	30	1.080	0.117	10.83

The presence of toluene did not materially alter these results. Hydrochloric acid, 0.5 per cent, after twenty hours, with toluene gave the following:

TABLE VI.

TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
20	0.890	0.117	13.14

When the bran is digested in dilute acetic acid the hydrolysis is greater than with the same strength hydrochloric acid, as shown by the following results:

TABLE VII.

SOLVENT	TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.2% acetic	3	1.21	0.695	57.03
0.2% acetic	24	1.23	1.050	85.54

The maximum activity of the enzyme, as shown by the greatest hydrolysis, was obtained by digesting bran for forty-eight hours in water and then adding 0.1 per cent hydrochloric acid and allowing it to stand for another twenty-four hours. Ten grams of bran were digested in 100 cc. of water for forty-eight hours and then 100 cc. of 0.2 per cent hydrochloric acid were added. After standing for twenty-four hours more it was filtered and 20 cc. of the filtrate were used for each determination.

TABLE VIII.

TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1.24	1.14	92.37



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### *Destruction of the enzyme phytase by heat.*

As is shown by the following experiments, the hydrolytic action of the enzyme is completely destroyed when bran is exposed to the action of boiling water or boiling dilute hydrochloric acid for a short time. Ten grams of bran were placed in an Erlenmeyer flask, and 100 cc. of boiling water poured over it. The solution was then heated for a few minutes until the water boiled. It was allowed to digest for twenty-four hours at room temperature. Another lot of bran was treated in the same way, but with 0.2 per cent hydrochloric acid instead of water. The resulting extracts could not be filtered. The whole was therefore diluted with 100 cc. of 2 per cent hydrochloric acid and allowed to stand for a few minutes in order to settle. Lots of 20 cc. of the liquid were then taken for each determination.

TABLE IX.

SOLVENT	TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Water	24	0.886	0.108	12.66
0.2% HCl	24	1.140	0.105	9.22

The hydrolytic action of the enzyme is likewise destroyed by exposing bran for a short time to 0.5 per cent hydrochloric acid or by digesting bran in dilute ammonia. Ten grams of bran were digested in 100 cc. of 0.5 per cent hydrochloric acid for one and one-half hours. The acid was then nearly neutralized with ammonia, leaving the solution faintly acid, and then standing for twenty-four hours. Ten grams of bran were digested in 100 cc. of 0.25 per cent ammonia for twenty-four hours. It was then acidified with dilute hydrochloric acid.

TABLE X.

SOLVENT	TIME	TOTAL PHOSPHORUS IN EXTRACT	INORGANIC PHOSPHORUS IN EXTRACT	TOTAL PHOSPHORUS AS INORGANIC
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.5% HCl	24	0.661	0.115	17.46
0.25% NH <sub>3</sub>	24	0.922	0.087	9.51

The results reported in this and the preceding paper will naturally change the interpretation of our previous investigations concerning the nature of the organic phosphorus compound of wheat bran. We have heretofore been under the impression that the substances which we have isolated from wheat bran, after digesting in 0.2 per cent hydrochloric acid, represented the compounds originally present in the bran. That this supposition is erroneous is evident, since the action of a bran extract on phytin gives rise to identical compounds.

A consideration of the results reported in Table V likewise shows that there is a wide difference between the action of 0.2 and 1.0 per cent hydrochloric acid on bran. In the first case the extract contains 0.925 per cent inorganic phosphorus, and in the second only 0.081 per cent.

These facts lead to the conclusion that the products which we have previously isolated from wheat bran represent only such intermediate compounds as have been formed by the action of the enzyme phytase upon the original phytin of the bran.

#### SUMMARY.

The results herein reported amplify and confirm the experiments of Suzuki, Yoshimura, and Takaishi, and of Plimmer concerning the presence of the enzyme phytase in wheat bran, which is capable of hydrolyzing the phytin with the production of inorganic phosphoric acid.

The maximum activity of the enzyme has been shown to occur in the presence of 0.1 per cent hydrochloric acid and 0.2 per cent acetic acid. With increasing concentration of the hydrochloric acid the activity rapidly diminishes, and with 0.5 per cent hydrochloric acid there is practically no hydrolysis of the organic phosphorus.

The enzyme is destroyed by boiling water and by boiling 0.2 per cent hydrochloric acid. It is also destroyed by a short exposure to 0.5 per cent hydrochloric acid and to 0.25 per cent ammonia.

It is shown that wheat bran normally contains about 0.1 per cent of inorganic phosphorus, which is equal to about 11 per cent of the total soluble phosphorus.



## CONCERNING PHYTIN IN WHEAT BRAN.

FOURTEENTH PAPER ON PHYTIN.

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### INTRODUCTION.

It has been shown in earlier reports<sup>1</sup> that the organic phosphorus compounds of wheat bran which have been isolated after digesting the bran in 0.2 per cent hydrochloric acid differ in composition and properties from phytin or inosite hexaphosphate. It has been found that under these conditions several organic phosphoric acids are obtained. Of these we have isolated and identified two; *viz.*, inosite monophosphate<sup>2</sup> and inosite triphosphate.<sup>3</sup>

Patten and Hart,<sup>4</sup> who first investigated the phosphorus compounds of wheat bran, came to the conclusion that the organic phosphoric acid in bran was identical with phytic acid, or the "anhydrooxymethylene diphosphoric acid" of Posternak. These authors isolated the acid preparation which they analyzed after digesting the bran in 0.2 per cent hydrochloric acid. We have shown conclusively, however, that when wheat bran is digested in this strength hydrochloric acid, the organic phosphorus compounds finally isolated are entirely different in composition from phytin or inosite hexaphosphate.

<sup>1</sup> R. J. Anderson: this *Journal*, xii, p. 447, 1912; xviii, p. 425, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 22, 1912, and No. 36, 1914.

<sup>2</sup> Anderson: this *Journal*, xviii, p. 441, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 36, 1914.

<sup>3</sup> Anderson: this *Journal*, xx, p. 463, 1915.

<sup>4</sup> A. J. Patten and E. B. Hart: *Am. Chem. Jour.*, xxxi, p. 566, 1904.

By the same method as above, *i.e.*, after digesting in 0.2 per cent hydrochloric acid, we have isolated inosite hexaphosphate from corn,<sup>5</sup> cottonseed meal,<sup>6</sup> and oats.<sup>7</sup> All these preparations were found to be identical with the inosite hexaphosphate prepared from commercial phytin.<sup>8</sup>

It would appear, then, as if wheat bran is the only one of all the various plants and seeds examined which does not contain inosite hexaphosphate. Instead, certain lower inosite phosphoric acids appear to be present. It seems difficult to explain why wheat bran should contain different inosite phosphoric acids from other plants.

The work of Suzuki, Yoshimura, and Takaishi,<sup>9</sup> and of Plimmer<sup>10</sup> on the presence of an enzyme in wheat bran which rapidly hydrolyzes phytin with formation of inorganic phosphoric acid, particularly in connection with the two preceding investigations, offered a key to the solution of this problem. The fact that the action of a wheat bran extract on commercial phytin yielded products identical with those which we have previously isolated from wheat bran itself, *viz.*, principally inosite triphosphate and inosite monophosphate, led to the theory that the organic phosphorus compound originally present in bran was probably hydrolyzed during the extraction with 0.2 per cent hydrochloric acid with formation of inorganic phosphoric acid and lower inosite phosphates. This opinion was fully confirmed by the results reported in the preceding paper. It is shown there that inorganic phosphates are liberated rapidly when wheat bran is digested in water, in 0.1 and in 0.2 per cent hydrochloric acid, as well as in 0.2 per cent acetic acid. This rapid formation of inorganic phosphoric acid from the organic phosphorus compound would

<sup>5</sup> Anderson: this *Journal*, xvii, p. 165, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>6</sup> Anderson: this *Journal*, xvii, p. 141, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>7</sup> Anderson: this *Journal*, xvii, p. 151, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>8</sup> Anderson: this *Journal*, xvii, p. 171, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>9</sup> U. Suzuki, K. Yoshimura, and M. Takaishi: *Bulletins of the College of Agriculture*, Tokyo, vii, pp. 503-512, 1907.

<sup>10</sup> R. H. A. Plimmer: *Biochem. Jour.*, vii, p. 43, 1913.

naturally preclude the possibility of isolating the original substance from extracts prepared by digesting the material in the above solutions. The only products possible of isolation would be such intermediate substances as had escaped complete hydrolysis by the enzyme. The previous determinations concerning the activity of the enzyme showed, however, that the use of hydrochloric acid stronger than 0.2 per cent materially reduced the amount of inorganic phosphate in the extract, and that the minimum quantity was present when the bran was extracted with 1 per cent hydrochloric acid.

Wheat bran was therefore extracted for five hours with 1 per cent hydrochloric acid and the organic phosphate in the extract isolated as a barium salt (compare experimental part). The substance then obtained had entirely different properties and composition from those obtained when wheat bran is digested in 0.2 per cent hydrochloric acid. After careful purification this salt crystallized in the same form and under the same conditions as the corresponding barium salts of inositol hexaphosphate. So far as crystal form, properties, and composition are concerned, there appears to be no difference between the substances isolated from wheat bran and the barium salts of phytic acid or inositol hexaphosphate obtained from other sources. We conclude, therefore, that all these materials, *viz.*, wheat bran, corn, oats, cottonseed meal, and commercial phytin, contain the same organic phosphates; namely, phytic acid or inositol hexaphosphate,  $C_6H_{18}O_{24}P_6$ .

This confirms the conclusions of Patten and Hart<sup>11</sup> as to the nature of the organic phosphorus compound of wheat bran. It is somewhat difficult to understand how these authors came to this conclusion, since they state that they had extracted the bran with 0.2 per cent hydrochloric acid, and under these conditions we have shown that phytic acid or inositol hexaphosphate is not obtained, but principally inositol triphosphate and some inositol monophosphate.

In view of the results reported in this as well as in the two preceding papers, it is evident that the compounds which we have previously isolated from wheat bran, *viz.*, inositol triphosphate and inositol monophosphate, do not represent the organic phos-

<sup>11</sup> Patten and Hart: *loc. cit.*

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<sup>11</sup> Patten and Hart: *loc. cit.*



phoric acids originally present in the bran, but that they are intermediate products formed from inosite hexaphosphate by the enzyme phytase during the extraction of the bran with the dilute acid.

#### EXPERIMENTAL PART.

The bran, 700 grams, was digested in five liters of 1 per cent hydrochloric acid for five hours. It was then strained through cheese-cloth and the liquid filtered. Barium hydroxide (Kahlbaum) was added to the filtrate until the reaction was alkaline. The precipitate was filtered and washed with water and then dissolved in about 3 per cent hydrochloric acid. The opalescent solution was filtered through charcoal and the filtrate precipitated by adding about an equal volume of alcohol. After standing over night the precipitate was filtered, washed in dilute alcohol, again dissolved in 3 per cent hydrochloric acid, and filtered through charcoal. A dilute solution of barium hydroxide was gradually added to the filtrate until a precipitate began to form. After standing over night the substance had separated out in semicrystalline form. It was filtered and washed in water, dissolved in 3 per cent hydrochloric acid, and precipitated by alcohol. After filtering and washing with dilute alcohol it was again dissolved in the dilute hydrochloric acid and precipitated by barium hydroxide.

The precipitate was still dark colored and it contained some impurities, not completely soluble in the dilute hydrochloric acid, apparently of colloidal nature, which could not be removed by filtration. In order to eliminate these impurities the barium precipitate was suspended in water and the barium removed with a slight excess of dilute sulphuric acid. The barium sulphate was filtered off and the filtrate precipitated with excess of copper acetate. The copper precipitate was filtered and washed free of sulphates with water. It was then suspended in water and decomposed with hydrogen sulphide. After filtering off the copper sulphide a clear and colorless solution of the free acid was obtained. By these various operations the oxalic acid had also been removed, as after nearly neutralizing with barium hydroxide and adding barium chloride, no precipitate or turbidity occurred. The solution was precipitated with barium hydroxide, filtered,

and washed in water. The substance was again twice precipitated with barium hydroxide from 3 per cent hydrochloric acid, and finally twice precipitated with alcohol from the same strength hydrochloric acid. After filtration the substance was washed free of chlorides with dilute alcohol, alcohol, and ether, and dried in vacuum over sulphuric acid. It was a snow-white semicrystalline powder weighing 11 grams. It was free from chlorides and inorganic phosphate and did not contain any bases except barium.

For analysis it was recrystallized as follows: 2 grams of the substance were dissolved in the minimum quantity of 2 per cent hydrochloric acid, the free acid was nearly neutralized with barium hydroxide, and the solution filtered. The clear filtrate was heated to boiling, when the substance separated as a heavy crystalline powder. This was filtered and washed in boiling hot water, and finally in alcohol and ether, and dried in the air. Yield, 1.5 grams. It was recrystallized a second time in the same manner except that 20 cc. of  $\frac{N}{7}$  barium chloride were added to the solution before boiling. After filtering, washing, and drying, as before, about 1.3 grams of substance were obtained. It consisted of fine, microscopic, needle-shaped crystals. It was free from chlorides and the nitric acid solution gave no precipitate with ammonium molybdate, showing that inorganic phosphate was absent.

It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.3771 gram substance lost 0.0385 gram  $H_2O$ .

0.1917 gram substance lost 0.0196 gram  $H_2O$ .

0.3386 gram substance gave 0.0361 gram  $H_2O$  and 0.0794 gram  $CO_2$ .

0.2571 gram substance gave 0.0269 gram  $H_2O$  and 0.0617 gram  $CO_2$ .

0.1715 gram substance gave 0.1218 gram  $BaSO_4$  and 0.1026 gram  $Mg_2P_2O_7$ .

Found: C = 6.39; H = 1.19; P = 16.67; Ba = 41.79 per cent.

C = 6.54; H = 1.17.

$H_2O$  = 10.20 and 10.22 per cent.

For heptabarium inosite hexaphosphate,  $(C_8H_{11}O_{24}P_6)_2Ba_7 = 2267$ .

Calculated: C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.

For 14  $H_2O$  calculated, 10.00 per cent.

*Preparation of the crystalline tribarium inosite hexaphosphate.*

This was prepared by dissolving 5 grams of the original substance in 2 per cent hydrochloric acid, nearly neutralizing with

dilute barium hydroxide, filtering, and adding alcohol gradually until the solution turned cloudy. It was then allowed to stand for two days at room temperature. The substance separated slowly in the form of globular masses or rosettes of microscopic needles. The crystal form was identical with that previously described for the tribarium inosite hexaphosphate. The substance was filtered and washed in dilute alcohol, alcohol, and ether, and dried in the air. It was recrystallized a second time by dissolving in the minimum quantity of 2 per cent hydrochloric acid, filtering, and adding alcohol until a slight permanent cloudiness remained. After standing for two days the substance had separated in the same form as before. It was filtered, washed free of chlorides with dilute alcohol, and then in alcohol and ether, and dried in the air. It was obtained as a snow-white crystalline powder. It was free from chlorides and inorganic phosphates.

For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

0.2922 gram substance lost 0.0334 gram  $H_2O$ .

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Found: C = 6.82; H = 1.25; P = 17.00; Ba = 39.09 per cent.

$H_2O$  = 11.43 per cent.

For tribarium inosite hexaphosphate,  $C_8H_{11}O_{11}P_6Ba_3$  = 1066.

Calculated: C = 6.75; H = 1.12; P = 17.44; Ba = 38.65 per cent.

For 2  $H_2O$  calculated, 11.90 per cent.

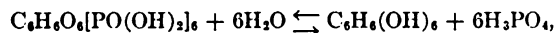
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The substance is first burned in the usual way in a current of oxygen, the combustion lasting about an hour. The calcium chloride tube and the potash bulb are then weighed. The increase in weight of the calcium chloride tube is taken as the correct weight of the water. The residue in the boat, which is dark colored from particles of unburned carbon, is powdered in an agate mortar with some recently fused potassium bichromate and again placed in the boat, the mortar being rinsed out with some more powdered bichromate. The whole is again burned in the usual way. The potassium bichromate fuses and oxidizes all the carbon in the residue. The increase in weight in the potash bulb is added to the first, giving the total carbon dioxide.

Since the barium salts described above agree in crystal form and composition with salts of inosite hexaphosphoric acid or phytic acid, we believe there can be no doubt that wheat bran contains the same phytin as other plants. We would have recognized this relation sooner if we had made a series of inorganic phosphorus determinations in wheat bran extracts prepared with water or dilute acids, such as is reported in a preceding paper. We believed, however, that since phytic acid could be isolated from cottonseed meal, oats, and corn, after digesting in 0.2 per cent hydrochloric acid, that the same procedure should also suffice in the case of wheat bran. Moreover, we were following the method of isolation recommended by previous investigators on this subject. The fact that inosite hexaphosphate is obtained from 0.2 per cent hydrochloric acid extracts of corn, oats, and cottonseed meal only proves that these materials, with the possible exception of oats, do not contain any enzyme of the nature of the phytase contained in wheat bran. In the case of the phytin preparations isolated from oats<sup>12</sup> we found a considerable amount of water-soluble barium salt of practically the same composition as the barium salts prepared from wheat bran after extracting with 0.2 per cent hydrochloric acid. We hope to investigate further this matter concerning the distribution of the enzyme phytase in various plants. We also intend to study more closely certain phases of the action of this enzyme, particularly

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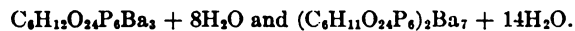
whether it is capable of inducing synthetic reactions. It appears not improbable that an equilibrium exists in the reaction



particularly at the stage when one-half of the phosphorus has been split off. The fact that the bulk of the substance ordinarily isolated from wheat bran, after digesting in 0.2 per cent hydrochloric acid, is inosite triphosphate, supports this view.

#### SUMMARY.

By digesting wheat bran in 1.0 per cent hydrochloric acid, which is sufficiently strong to destroy the enzyme, phytase, it is possible to isolate from the extract crystalline barium salts of the following composition:



These salts are identical with the tribarium phytate and heptabarium phytate obtained from oats, corn, cottonseed meal, and commercial phytin. All these materials contain, therefore, the same organic phosphorus compound; *viz.*, phytic acid or inosite hexaphosphate,  $\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$ .

The author acknowledges with pleasure his indebtedness to Dr. R. H. A. Plimmer for many suggestions which have been of great assistance in carrying out the work reported in this and the two preceding papers.

**A METHOD FOR THE DECOMPOSITION OF THE PROTEINS OF THE THYROID, WITH A DESCRIPTION OF CERTAIN CONSTITUENTS.<sup>1, 2</sup>**

By E. C. KENDALL.

(From the Mayo Clinic, Rochester, Minn.)

(Received for publication, January 28, 1915.)

In 1895 Baumann<sup>3</sup> reported the discovery of iodine in the thyroid, and during the succeeding years there has been an ever increasing interest in the study of the chemical constituents and physiologic activity of the gland. As early as 1896 Gottlieb<sup>4</sup> made the statement that from clinical and experimental observations it is evident that there must be more than one physiologically active compound in the thyroid, but up to the present time no compound of known chemical constitution has been isolated which produces physiologic effects similar to those of desiccated thyroid. Attempts to isolate the active principle have resulted in preparations which may be divided into two classes: first, those secured by separation of products of protein nature without decomposition or destruction of the protein molecule; and, second, those obtained by means of hydrolysis of the protein and the subsequent separation of the decomposition products. In the first class of preparations are Oswald's<sup>5</sup> thyroglobulin and

<sup>1</sup> Read before the Section of Biological Chemists of the Federation of American Scientific Societies, St. Louis, Dec. 28, 1914.

<sup>2</sup> This work was begun and in most part completed in the Pathological Department of St. Luke's Hospital, New York. I wish to thank Dr. F. C. Wood for the opportunity of carrying on the investigation in that Institution.

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By the same method as above, *i.e.*, after digesting in 0.2 per cent hydrochloric acid, we have isolated inosite hexaphosphate from corn,<sup>5</sup> cottonseed meal,<sup>6</sup> and oats.<sup>7</sup> All these preparations were found to be identical with the inosite hexaphosphate prepared from commercial phytin.<sup>8</sup>

It would appear, then, as if wheat bran is the only one of all the various plants and seeds examined which does not contain inosite hexaphosphate. Instead, certain lower inosite phosphoric acids appear to be present. It seems difficult to explain why wheat bran should contain different inosite phosphoric acids from other plants.

The work of Suzuki, Yoshimura, and Takaishi,<sup>9</sup> and of Plimmer<sup>10</sup> on the presence of an enzyme in wheat bran which rapidly hydrolyzes phytin with formation of inorganic phosphoric acid, particularly in connection with the two preceding investigations, offered a key to the solution of this problem. The fact that the action of a wheat bran extract on commercial phytin yielded products identical with those which we have previously isolated from wheat bran itself, *viz.*, principally inosite triphosphate and inosite monophosphate, led to the theory that the organic phosphorus compound originally present in bran was probably hydrolyzed during the extraction with 0.2 per cent hydrochloric acid with formation of inorganic phosphoric acid and lower inosite phosphates. This opinion was fully confirmed by the results reported in the preceding paper. It is shown there that inorganic phosphates are liberated rapidly when wheat bran is digested in water, in 0.1 and in 0.2 per cent hydrochloric acid, as well as in 0.2 per cent acetic acid. This rapid formation of inorganic phosphoric acid from the organic phosphorus compound would

<sup>5</sup> Anderson: this *Journal*, xvii, p. 165, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>6</sup> Anderson: this *Journal*, xvii, p. 141, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>7</sup> Anderson: this *Journal*, xvii, p. 151, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>8</sup> Anderson: this *Journal*, xvii, p. 171, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 32, 1914.

<sup>9</sup> U. Suzuki, K. Yoshimura, and M. Takaishi: *Bulletins of the College of Agriculture*, Tokyo, vii, pp. 503-512, 1907.

<sup>10</sup> R. H. A. Plimmer: *Biochem. Jour.*, vii, p. 43, 1913.

naturally preclude the possibility of isolating the original substance from extracts prepared by digesting the material in the above solutions. The only products possible of isolation would be such intermediate substances as had escaped complete hydrolysis by the enzyme. The previous determinations concerning the activity of the enzyme showed, however, that the use of hydrochloric acid stronger than 0.2 per cent materially reduced the amount of inorganic phosphate in the extract, and that the minimum quantity was present when the bran was extracted with 1 per cent hydrochloric acid.

Wheat bran was therefore extracted for five hours with 1 per cent hydrochloric acid and the organic phosphate in the extract isolated as a barium salt (compare experimental part). The substance then obtained had entirely different properties and composition from those obtained when wheat bran is digested in 0.2 per cent hydrochloric acid. After careful purification this salt crystallized in the same form and under the same conditions as the corresponding barium salts of inositol hexaphosphate. So far as crystal form, properties, and composition are concerned, there appears to be no difference between the substances isolated from wheat bran and the barium salts of phytic acid or inositol hexaphosphate obtained from other sources. We conclude, therefore, that all these materials, *viz.*, wheat bran, corn, oats, cottonseed meal, and commercial phytin, contain the same organic phosphates; namely, phytic acid or inositol hexaphosphate,  $C_6H_{18}O_{24}P_6$ .

This confirms the conclusions of Patten and Hart<sup>11</sup> as to the nature of the organic phosphorus compound of wheat bran. It is somewhat difficult to understand how these authors came to this conclusion, since they state that they had extracted the bran with 0.2 per cent hydrochloric acid, and under these conditions we have shown that phytic acid or inositol hexaphosphate is not obtained, but principally inositol triphosphate and some inositol monophosphate.

In view of the results reported in this as well as in the two preceding papers, it is evident that the compounds which we have previously isolated from wheat bran, *viz.*, inositol triphosphate and inositol monophosphate, do not represent the organic phos-

<sup>11</sup> Patten and Hart: *loc. cit.*



phoric acids originally present in the bran, but that they are intermediate products formed from inosite hexaphosphate by the enzyme phytase during the extraction of the bran with the dilute acid.

#### EXPERIMENTAL PART.

The bran, 700 grams, was digested in five liters of 1 per cent hydrochloric acid for five hours. It was then strained through cheese-cloth and the liquid filtered. Barium hydroxide (Kahlbaum) was added to the filtrate until the reaction was alkaline. The precipitate was filtered and washed with water and then dissolved in about 3 per cent hydrochloric acid. The opalescent solution was filtered through charcoal and the filtrate precipitated by adding about an equal volume of alcohol. After standing over night the precipitate was filtered, washed in dilute alcohol, again dissolved in 3 per cent hydrochloric acid, and filtered through charcoal. A dilute solution of barium hydroxide was gradually added to the filtrate until a precipitate began to form. After standing over night the substance had separated out in semicrystalline form. It was filtered and washed in water, dissolved in 3 per cent hydrochloric acid, and precipitated by alcohol. After filtering and washing with dilute alcohol it was again dissolved in the dilute hydrochloric acid and precipitated by barium hydroxide.

The precipitate was still dark colored and it contained some impurities, not completely soluble in the dilute hydrochloric acid, apparently of colloidal nature, which could not be removed by filtration. In order to eliminate these impurities the barium precipitate was suspended in water and the barium removed with a slight excess of dilute sulphuric acid. The barium sulphate was filtered off and the filtrate precipitated with excess of copper acetate. The copper precipitate was filtered and washed free of sulphates with water. It was then suspended in water and decomposed with hydrogen sulphide. After filtering off the copper sulphide a clear and colorless solution of the free acid was obtained. By these various operations the oxalic acid had also been removed, as after nearly neutralizing with barium hydroxide and adding barium chloride, no precipitate or turbidity occurred. The solution was precipitated with barium hydroxide, filtered,

and washed in water. The substance was again twice precipitated with barium hydroxide from 3 per cent hydrochloric acid, and finally twice precipitated with alcohol from the same strength hydrochloric acid. After filtration the substance was washed free of chlorides with dilute alcohol, alcohol, and ether, and dried in vacuum over sulphuric acid. It was a snow-white semicrystalline powder weighing 11 grams. It was free from chlorides and inorganic phosphate and did not contain any bases except barium.

For analysis it was recrystallized as follows: 2 grams of the substance were dissolved in the minimum quantity of 2 per cent hydrochloric acid, the free acid was nearly neutralized with barium hydroxide, and the solution filtered. The clear filtrate was heated to boiling, when the substance separated as a heavy crystalline powder. This was filtered and washed in boiling hot water, and finally in alcohol and ether, and dried in the air. Yield, 1.5 grams. It was recrystallized a second time in the same manner except that 20 cc. of  $\frac{N}{7}$  barium chloride were added to the solution before boiling. After filtering, washing, and drying, as before, about 1.3 grams of substance were obtained. It consisted of fine, microscopic, needle-shaped crystals. It was free from chlorides and the nitric acid solution gave no precipitate with ammonium molybdate, showing that inorganic phosphate was absent.

It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.3771 gram substance lost 0.0385 gram  $H_2O$ .

0.1917 gram substance lost 0.0196 gram  $H_2O$ .

0.3386 gram substance gave 0.0361 gram  $H_2O$  and 0.0794 gram  $CO_2$ .

0.2571 gram substance gave 0.0269 gram  $H_2O$  and 0.0617 gram  $CO_2$ .

0.1715 gram substance gave 0.1218 gram  $BaSO_4$  and 0.1026 gram  $Mg_2P_2O_7$ .

Found: C = 6.39; H = 1.19; P = 16.67; Ba = 41.79 per cent.

C = 6.54; H = 1.17.

$H_2O$  = 10.20 and 10.22 per cent.

For heptabarium inosite hexaphosphate,  $(C_6H_{11}O_{24}P_6)_2Ba_7 = 2267$ .

Calculated: C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.

For 14  $H_2O$  calculated, 10.00 per cent.

*Preparation of the crystalline tribarium inosite hexaphosphate.*

This was prepared by dissolving 5 grams of the original substance in 2 per cent hydrochloric acid, nearly neutralizing with

dilute barium hydroxide, filtering, and adding alcohol gradually until the solution turned cloudy. It was then allowed to stand for two days at room temperature. The substance separated slowly in the form of globular masses or rosettes of microscopic needles. The crystal form was identical with that previously described for the tribarium inosite hexaphosphate. The substance was filtered and washed in dilute alcohol, alcohol, and ether, and dried in the air. It was recrystallized a second time by dissolving in the minimum quantity of 2 per cent hydrochloric acid, filtering, and adding alcohol until a slight permanent cloudiness remained. After standing for two days the substance had separated in the same form as before. It was filtered, washed free of chlorides with dilute alcohol, and then in alcohol and ether, and dried in the air. It was obtained as a snow-white crystalline powder. It was free from chlorides and inorganic phosphates.

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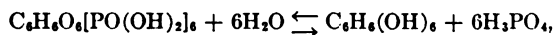
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The substance is first burned in the usual way in a current of oxygen, the combustion lasting about an hour. The calcium chloride tube and the potash bulb are then weighed. The increase in weight of the calcium chloride tube is taken as the correct weight of the water. The residue in the boat, which is dark colored from particles of unburned carbon, is powdered in an agate mortar with some recently fused potassium bichromate and again placed in the boat, the mortar being rinsed out with some more powdered bichromate. The whole is again burned in the usual way. The potassium bichromate fuses and oxidizes all the carbon in the residue. The increase in weight in the potash bulb is added to the first, giving the total carbon dioxide.

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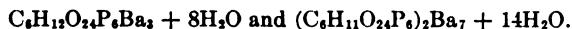
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#### SUMMARY.

By digesting wheat bran in 1.0 per cent hydrochloric acid, which is sufficiently strong to destroy the enzyme, phytase, it is possible to isolate from the extract crystalline barium salts of the following composition:



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**A METHOD FOR THE DECOMPOSITION OF THE PRO-  
TEINS OF THE THYROID, WITH A DESCRIPTION  
OF CERTAIN CONSTITUENTS.<sup>1,2</sup>**

BY E. C. KENDALL.

(From the Mayo Clinic, Rochester, Minn.)

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In 1895 Baumann<sup>3</sup> reported the discovery of iodine in the thyroid, and during the succeeding years there has been an ever increasing interest in the study of the chemical constituents and physiologic activity of the gland. As early as 1896 Gottlieb<sup>4</sup> made the statement that from clinical and experimental observations it is evident that there must be more than one physiologically active compound in the thyroid, but up to the present time no compound of known chemical constitution has been isolated which produces physiologic effects similar to those of desiccated thyroid. Attempts to isolate the active principle have resulted in preparations which may be divided into two classes: first, those secured by separation of products of protein nature without decomposition or destruction of the protein molecule; and, second, those obtained by means of hydrolysis of the protein and the subsequent separation of the decomposition products. In the first class of preparations are Oswald's<sup>5</sup> thyroglobulin and

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iodine-free nucleoprotein. Other investigators have prepared similar proteins from the thyroid. These products, which are original protein compounds unchanged in chemical nature, still retain their activity, as shown in the treatment of symptoms of cretinism and myxedema. In the second class of preparations, Baumann's iodothylin, containing about 9 per cent of iodine, is the result of the decomposition of the proteins with sulphuric acid. The iodothylin so obtained is about 4 per cent of the total weight of the dried thyroid. It has some physiologic activity, but the original claim of Baumann that it is *the* physiologically active principle of the thyroid has long since been disproved.

Other decomposition products have been obtained by Hutchinson,<sup>6</sup> working with pepsin and trypsin on the thyroid proteins. Hutchinson separated in this way a product containing 3.6 per cent of iodine.

Another means of throwing light on the nature of the iodine compound has been to prepare various iodine compounds and test the physiologic activity of these. Di-iodotyrosine, tetra-iodo-histidine, tri-iodo imidazol, iodized tryptophane, and iodized phenylalanine, and other organic compounds have been tested in this way,<sup>7</sup> but no compound has been found which produces effects similar to those of desiccated thyroid.

Four years ago I took up the investigation of the chemical constituents of the thyroid. The object of the work was to isolate in pure form one or more chemical compounds which possess physiologic activity. Dialysis was used as a preliminary study of the proteins of the gland and the nature of iodine combination. Desiccated thyroid either in suspension or dissolved in dilute alkali will lose less than 5 per cent of its total iodine by dialysis in a collodion sac against running water. Experiments

<sup>6</sup> R. Hutchinson: The Chemistry of the Thyroid Gland and the Nature of its Active Constituent, *Jour. Physiol.*, xx, pp. 474-495, 1896. Also, Further Observations on the Chemistry and Action of the Thyroid Gland, *ibid.*, xxiii, pp. 178-189, 1898-99.

<sup>7</sup> O. von Fürth and K. Schwarz: Über die Einwirkung des Jodothyrlins auf den Zirkulationsapparat, *Arch. f. d. ges. Physiol.*, cxxiv, pp. 113-156, 1908. S. Strouse and C. Voegtlin: Studies Concerning the Iodine-Containing Principle of the Thyroid Gland, *Jour. Pharm. and Exper. Therap.*, i, pp. 122-133, 1909-10. F. C. Koch: On the Nature of the Iodine-Containing Complex in Thyreoglobulin, *this Journal*, xiv, 101-116, 1913.

were then made varying the temperature and acidity of the dialysate. These results showed that increase in temperature and acidity favored dialysis of the iodine compound, and as much as 40 per cent could be made to pass through the sac in this way. Attempts were then made to alter the nature of the proteins to see the attending influence on dialysis of the iodine. Boiling in strong sodium hydroxide allowed 80 per cent of the iodine to pass the dialyzing sac. Boiling with sodium hydroxide and hydrogen peroxide allowed 94 per cent to pass.

These results showed a decomposition of the protein and a probable splitting off of iodine in the inorganic form. As such vigorous treatment would undoubtedly destroy physiologic activity, further experiments were carried out to find some treatment which would break down the complex proteins into simpler products without destruction of the compounds so obtained. Alcohol was tried as a medium for the carrying out of such treatment. Alcohol saturated with hydrochloric acid gas was tried as a hydrolytic agent, but no satisfactory cleavage resulted from its use. Hydrolysis with sodium hydroxide in alcohol was then tried; and it was found that this method produced a cleavage different from any of the others. 75 per cent of the iodine was dialyzable, but it was easily shown that the iodine was not split off as sodium iodide, but still existed in organic combination. After it was shown that sodium hydroxide in alcohol altered the nature of the protein to a marked degree, dialysis, as a criterion of the nature of the iodine combination, was discontinued and a detailed study of the chemical properties of these products of hydrolysis was begun.

No specific precipitant was found for the iodine compound in either an alcoholic or aqueous solution. After many attempts to find such a reagent it became apparent that the iodine was present in two different forms of organic combination. About 50 per cent of the total iodine was soluble in acid, and 50 per cent was insoluble. As the solubility in acids effected a separation between the two apparently different iodine compounds, this treatment was used as the first step in the separation of the products of hydrolysis. Those compounds insoluble in acid are designated constituents of Group A, and those soluble, constituents of Group B.



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All the constituents of Group B are easily dialyzable. Saturation of a solution of B with ammonium sulphate produces a sticky, tarry precipitate which evidently consists of amino-acid complexes, and carries down with it about 80 per cent of the iodine in B, showing that it is still present in organic combination.

Among the constituents of B not precipitated with ammonium sulphate is a compound that reduces alkaline mercury and silver salts. Evaporation of the solution to dryness with sodium hydroxide does not affect its reducing power, hence the reduction of the silver cannot be due to ammonium compounds. This reducing compound has been designated R.

The iodine compound in B is precipitated to a large extent with mercury sulphate, and almost quantitatively with silver sulphate in the presence of magnesium oxide. A large percentage of the iodine is split off by this treatment. Oxidizing agents, even copper acetate, also easily split off the iodine from its organic combination.

After establishing the general chemical properties of Group B, efforts were directed to a more extended study of Group A. The most striking chemical property of A is its acidic nature. All the constituents of A are easily soluble in dilute alkali or ammonia, and are reprecipitated by any acid.

Experiments with organic solvents showed that uncombined sulphur, fatty acids, and about 10 per cent of the iodine in A is soluble in ether. The fatty acids doubtless came from the fats which were saponified by the alkaline alcohol, and the sulphur probably resulted from the decomposition of cystine. Further experiments showed that the solubility of the iodine in organic solvents varies greatly, it being least soluble in petroleum ether. The second step, therefore, for the purification of A, is the removal of fatty acids and sulphur by extraction with petroleum ether. The product thus obtained contains about 4 per cent of iodine, and this preparation may be dissolved in alkali and reprecipitated with acids without appreciable loss of its total iodine. This treatment slowly removes some constituents containing nitrogen, but no iodine, so that the percentage of iodine in A may thus be increased to about 6 per cent.

This preparation is a dark brown powder insoluble in water and acids, easily soluble in dilute alkali and ammonia. Its alkaline

solution is precipitated by copper hydroxide, and to a large extent by barium, calcium, and magnesium salts. It is almost entirely soluble in ethyl acetate, but by partial extraction with this solvent it is possible to separate A into two fractions. In the ethyl acetate soluble portion of A, the percentage of iodine is increased to 13 or 14 per cent, but the ethyl acetate insoluble fraction contains only 1.5 per cent iodine.

Except for the solubility in ether, the general chemical properties of A closely resemble those of a fatty acid. Upon further hydrolysis of purified, fatty acid-free A by means of sodium hydroxide and a high temperature, it was found that lauric acid and tryptophane are split off.<sup>8</sup>

Having separated the chemical constituents of the thyroid into several fractions which have different chemical properties, it seemed desirable to test the physiologic activity of these different products.<sup>9</sup>

For the testing of thyroid activity there are two methods: first, by relieving symptoms of hypothyroidism as found in myxedema and cretinism; second, by the production of symptoms of hyperthyroidism similar to those of exophthalmic goiter. Testing by the first method, it was found that B has a specific action on the skin. The dry scaly skin was changed to a moist normal condition by the use of B alone. Also, certain subjective symptoms were entirely relieved. These were soreness of bones and joints, and heat flashes over the skin. Muscle cramps were relieved and prevented by the compound R. When B was tested by the second method, it was found that no toxic symptoms could be produced by any of the constituents of B. Many tests carried on over a long period have failed to produce any toxic effect from B given intravenously, subcutaneously, or by mouth.

Testing A by the first method, it was found that subnormal pulse and temperature were raised to normal, metabolism was

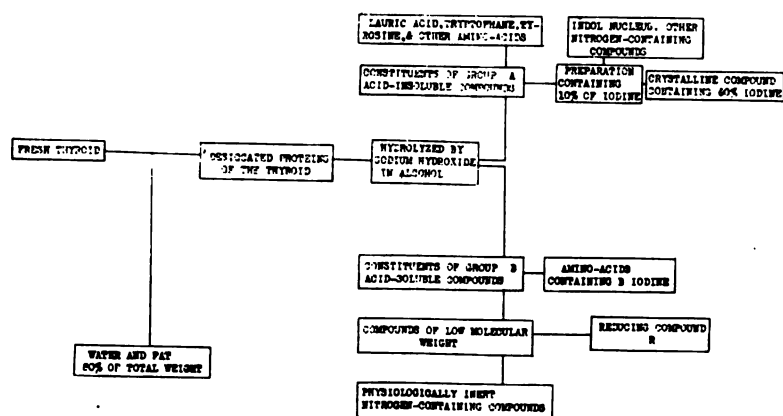
<sup>8</sup> The purification of A and the isolation of a crystalline compound containing 60 per cent of iodine will be described in another paper.

<sup>9</sup> In this paper these will be described briefly, but a detailed account of the methods employed and results obtained on a long series of experiments on man and dogs will appear in another publication.

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increased as shown by increase in nitrogen excretion and decrease in weight, and the mentality in cases of myxedema was brightened.

Testing A by the second method, toxic symptoms are produced by the administration of A. When injected subcutaneously in animals there is at first no effect on either the pulse rate or blood pressure. After twenty-four to thirty-six hours the dog appears restless, has a slight increase in temperature, and a decided increase in pulse rate. If a series of injections is given on successive days, these symptoms are aggravated and after two or three injections they are accompanied by a distinct tremor, loss of weight, and severe diarrhea. On the fourth or fifth day of



injection the pulse rate is between 200 and 300, and all the other symptoms continue with increased severity. This condition is followed by a sudden drop in the pulse rate, and continued injections at this time will not make the pulse rate return to its former high level, although the nervousness and tremor, loss of weight, and diarrhea continue without decrease in their severity. The dog in this condition presents a picture very similar to that of exophthalmic goiter. If the injections are now discontinued, all symptoms rapidly disappear and the dog will return to its former weight. The change in the pulse rate is interesting at this time, as it not only returns to normal but becomes much lower than before the injections had been given. These experiments have been repeated many times, giving practically the same result,

and show that A produces toxic symptoms similar to those of hyperthyroidism.

After it was found that the pulse rate could not be maintained at a very high figure for more than a few days, although the injections were continued, the question arose as to whether or not the animal developed a tolerance for the compound acting on the heart. If an immunity did result, it should be able to counteract the effects of a second series of injections given immediately after the animal returned to normal. To decide this question the animal was given a series of injections on several successive days, and after a marked toxic condition was obtained the animal was allowed to recover. After an interval of several days a second series of injections exactly similar to the first was given. Under these conditions the nervousness, tremor, and diarrhea appeared to be about as severe, but the pulse rate was much slower in responding to the injections, and at no time reached as high a figure as during the first series. There was no sudden drop in the pulse rate as before, and it did not return to normal for a longer period, the animal appearing to experience greater difficulty in throwing off the effect of the injections. After another interval, during which the animal returned to normal except in weight, a third series of injections was given similar to the first and second. All the symptoms were again produced, the animal losing weight very rapidly. The pulse appeared to be but slightly increased, but the least excitement or exertion would send it up to 280 or 300. The animal seemed to be even less capable of resisting the toxic effect than before.

From the results we may draw the conclusion that the toxic compound does not produce symptoms in a rapid and direct manner. It requires several hours before any effect is noticeable, and the symptoms continue for some time after stopping the injections. There is apparently some form of tolerance established in regard to the effect on the heart, but long continued injections slowly exhaust the body's resisting power.

Further experiments showed that for the production of these toxic symptoms it is necessary for the A injected to contain iodine, and the severity of the symptoms depends upon the amount of iodine injected. B iodine injected in equal amount produces no apparent effect and will not produce tolerance to a

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subsequent injection of A iodine. Iodine in the form of potassium iodide injected in amount five times that used for testing A produced no increase in the pulse rate. A prepared from desiccated thyroid or colloid goiters low in iodine is physiologically inert.

### *Details of the method for alkaline hydrolysis of proteins of the thyroid.*

The preparation of finely powdered desiccated thyroid, as fat-free as possible, is added to 90 per cent ethyl alcohol containing 1 per cent of sodium hydroxide, in the proportions of 2.5 grams of thyroid per 100 cc. of alcohol. The powder is insoluble and settles to the bottom of the container. The alcohol is now boiled for forty-eight hours under a reflux condenser. During the heating, ammonia is given off which amounts to about 8 per cent of the total nitrogen. At the end of this time the alcohol has dissolved the greater part of the powder and has a dark brown color.

The hot alcoholic solution is now filtered with suction. There is a sticky, tarry residue in the bottom of the flask which contains about 9 per cent of the total nitrogen, 80 per cent of the total phosphorus, and 7 per cent of the total iodine.

The alcohol solution is diluted with water until there is about 75 per cent of alcohol present, and the sodium hydroxide is neutralized with carbon dioxide or sulphuric acid. If sulphuric acid is used, the alcohol is cooled until the sodium sulphate crystallizes out. This is filtered off and the alcohol is then distilled, leaving a water solution of the split products. If carbon dioxide is used the alcohol is distilled immediately, as the sodium carbonate will not separate satisfactorily. The last traces of alcohol are removed by heating in an evaporating dish on the water bath. The water solution on cooling will solidify to a gelatinous mass if there is much fatty acid present.

The solution is diluted to about 100 cc. for each 20 to 25 grams of original thyroid used, and is acidified with 20 per cent sulphuric acid. This produces a precipitate. The acidification is continued until the addition of more acid ceases to cause a precipitate.

The solution is allowed to stand in the cold for several hours,—generally over night. The precipitate is filtered without suction and is washed with a little water. This now is the first preparation of A. It is allowed to dry on absorbent paper and then is completely desiccated *in vacuo* over sulphuric acid.

The filtrate is B. It is neutralized with sodium carbonate evaporated to small volume, alcohol is added, and the sodium sulphate crystallized out. The alcohol is distilled off and the resulting solution either desiccated or sterilized for therapeutic use. 15 to 20 per cent alcohol may be used as a preservative.

The dry A is extracted with petroleum ether, dissolved in dilute sodium hydroxide, and reprecipitated with sulphuric acid. By warming the solution, after addition of the acid, to about 60° and then cooling to about 10°, the precipitate changes to a sandy, finely divided form easily filtered. A in this form is fat-free, and amounts to about 5 per cent of the total weight of thyroid taken. Its percentage of iodine is about ten times as much as that of the starting material, and for normal glands is equal to about 50 per cent of the total iodine.

The distribution of nitrogen is as follows:

B contains 74 per cent of the total nitrogen.

A contains 9 per cent of the total nitrogen.

Alcohol residue contains 9 per cent of the total nitrogen.

Liberated ammonia contains 8 per cent of the total nitrogen.



## THE UREA CONTENT OF HUMAN SPINAL FLUID AND BLOOD.

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(Received for publication, February 5, 1915.)

It has long been realized that urea passes with peculiar ease through the membranes of the animal body, and that in consequence it penetrates into all the fluids of the organism.<sup>1</sup> The recent work of Marshall and Davis has given especially striking evidence of the rapidity with which urea distributes itself and attains approximately equal concentration throughout most of the tissues and fluids of the body.<sup>2</sup>

The necessity of drawing samples of blood and spinal fluid for clinical examination while following cases of tabes has offered the opportunity of testing in living human subjects the rule of equal distribution of urea among the different body fluids. Thirty-two determinations of both fluids from fifteen different patients were made. The samples of blood and spinal fluid were drawn within a few minutes of each other and were analyzed at once. The urea was determined by the Van Slyke and Cullen modification of Marshall's urease method.<sup>3</sup>

As will be seen from the table, in 63 per cent of the determinations the difference between the urea content of the blood and that of the spinal fluid was less than 2 mgm. per 100 cc. The greatest difference was 11 mgm. per 100 cc.

The urea values varied from 20 to 42, and from 22 to 46 mgm. of urea per 100 cc. of serum and spinal fluid respectively; all lie within the possible range of normal variation.

The occasional difference between spinal fluid and blood serum may be due to the rapid rise and fall of blood urea in different stages of protein digestion. From the nature of the process of

<sup>1</sup> C. Achard. Le rôle de l'urée en pathologie. *L'Oeuvre Médico-Chirurgicale*, lxx, p. 197, 1912.

<sup>2</sup> E. K. Marshall, Jr., and D. M. Davis: this *Journal*, xviii, p. 53, 1914. Especial concentration was noted only in the kidney.

<sup>3</sup> D. D. Van Slyke and G. E. Cullen: *ibid.*, xix, p. 211, 1914.



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secretion of spinal fluid<sup>4</sup> one would expect the changes in its urea content to lag somewhat behind those of the blood.

The results are in accordance with the already well founded view that the animal tissues are, in general, osmotically permeable to urea, which therefore tends to reach the same level of concentration in the different body fluids.

CASE	DATE (1914)	BLOOD UREA PER 100 CC.	SPINAL FLUID UREA PER 100 CC.
		mgm.	mgm.
1701	3/3	27	27
	3/17	28	30
	3/31	24	31
	4/7	40	32
	4/14	35	37
1822	3/3	29	28
	3/17	25	25
	3/31	20	27
	4/14	27	28
1873	2/27	36	34
	3/17	40	46
	4/3	22	22
	4/17	36	36
1794	2/27	28	27
	3/27	28	28
1111	4/3	24	26
	4/17	23	23
1098	2/26	32	28
	4/3	22	22
980	2/27	36	34
	3/27	31	27
1141		32	29
883		32	28
1699	3/3	27	25
	3/17	30	28
	3/31	22	24
	4/14	27	30
903		25	30
1177		29	27
1211		23	29
1862		42	31
1186		28	26
Average		29	29

<sup>4</sup> H. Cushing, L. H. Weed, and P. Wegefarth: *Jour. Med. Research*, xxxi, p. 1, 1914.

## MERCURY DERIVATIVES OF AROMATIC AMINES.

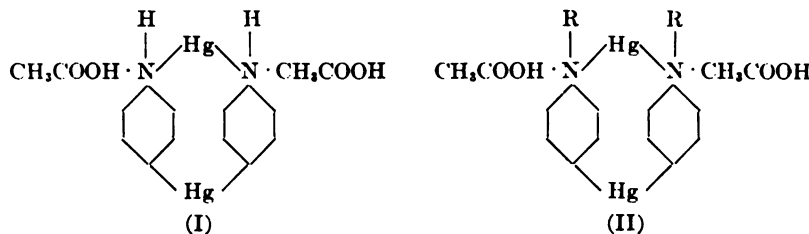
### I. CONTRIBUTION TO THE STRUCTURE OF PRIMARY AND SECONDARY *p*-AMINOPHENYLMERCURIC COMPOUNDS.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 4, 1915.)

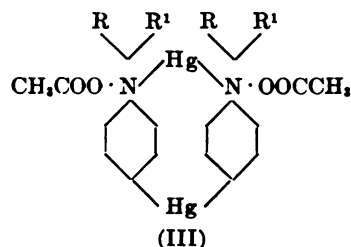
The pioneer work of Pesci<sup>1</sup> and the later investigations of Dimroth<sup>2</sup> on the synthesis and properties of mercuriated aromatic amines have added greatly to our knowledge of organometallic compounds. At the time, the exact structure of the mercury compounds obtained by the interaction of mercuric salts and aromatic amines was the subject of considerable dispute between the two workers. Pesci ascribed to the products obtained from primary and secondary amines the dimolecular formulas (I) and (II) respectively, basing his opinion chiefly on the fact that when these substances are heated with sodium sulphide or thiosulphate solutions one-half of their mercury is removed as mercuric sulphide with the formation of mercuridiaminodiaryls. This behavior, according to Pesci, indicates that one of the mercury atoms is more loosely bound (*i.e.*, on the nitrogen atom) than the other, which is firmly held between the two nuclei. Dimroth, on the other hand, pointed out that this reaction could be as well



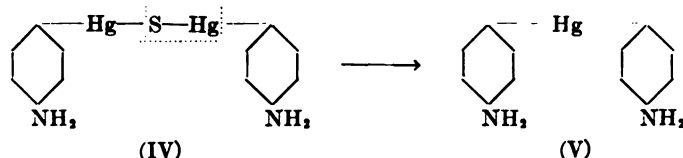
<sup>1</sup> Pesci: summarized in *Gazz. chim. ital.*, xxviii, pt. ii, p. 436, 1898. *Ztschr. f. anorg. Chem.*, xv, p. 208, 1897; xvii, p. 276, 1897-98.

<sup>2</sup> Dimroth: *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 2154, 1898; xxxii, p. 758, 1899; xxxv, p. 2038, 2853, 1902.

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explained on the basis of a monomolecular formula according to the following scheme. Two molecules of *p*-aminophenylmercuric acetate react with sodium sulphide yielding *p*-aminophenylmercuric sulphide (IV). This compound is unstable and splits off mercuric sulphide, as indicated by the dotted line, with the formation of *p*-mercuridianiline (V).



At the same time, owing to the close analogy of the amino derivatives to the compounds obtained by the treatment of aromatic hydrocarbons, nitro compounds, phenols, and acids with mercuric salts, substances which can be represented only on the basis of the monomolecular type, Dimroth was led to consider the products obtained from amines as also monomolecular. To carry greater conviction, he demonstrated the identity of the chloride of the product derived from dimethylaniline and mercuric acetate, believed by Pesci to be (III), with the substance obtained by the action of mercuric chloride on mercuridi-*p*-dimethylaniline. Since the method of preparation of the latter is similar to that of the undoubtedly monomolecular phenylmercuric chloride obtained from mercuridiphenyl and mercuric chloride, it must, according to Dimroth, have the monomolecular formula,  $\text{Me}_2\text{NC}_6\text{H}_4\text{HgCl}$ . Furthermore, it follows by analogy that in the case of the primary and secondary amino derivatives, in which the mercury undergoes the same reactions as in the tertiary compound, the monomolecular structure must also be accepted. The weakness of this "proof" was pointed out by Pesci,<sup>3</sup> who held to

<sup>3</sup> Pesci: *Ztschr. f. anorg. Chem.*, xxxii, p. 227, 1902.

his original views without adducing any additional evidence that was not immediately controverted by Dimroth.<sup>4</sup> The monomolecular formula has been accepted by all subsequent workers, since the clear reasoning of Dimroth scarcely admits of any other interpretation.

It seems, however, to the present writers that a more direct proof is available, and as a preliminary phase in a thorough study of certain mercuriated aromatic amines and their derivatives, we desire to present the following as proof of the monomolecular structure of these compounds.

It is obvious that if *p*-aminophenylmercuric acetate is monomolecular it must function as a primary amine, and not as in (I); likewise *p*-methylanilphenylmercuric acetate must function as a secondary amine. The following work demonstrates that these substances actually behave thus, forming, in the first case, azo derivatives and an azomethine; and in the second case yielding a nitroso derivative. A search of the literature revealed only a single case of a mercuriated amino compound behaving beyond doubt as a primary amine; viz., 3-methyl-4-aminophenylmercuric chloride, which forms a *diacetyl* derivative, the significance of which appears to have been overlooked by its discoverers.<sup>5</sup> It is, of course, evident that formula (I) admits of the preparation of monoacyl derivatives, so that these have no bearing on the question.

We are at present engaged in the development of the field of aminophenylmercuric compounds by making use of the usual transformations to which primary, secondary, and tertiary amines may be subjected, not only in the series of the aminophenylmercuric salts, but also in that of the mercuridiaminodiaryls.

#### EXPERIMENTAL.

##### 1. Derivatives of *p*-aminophenylmercuric acetate.

Although Dimroth states that this substance is easily soluble in mineral acids,<sup>6</sup> none of our many preparations of the compound showed this property.

<sup>4</sup> Dimroth: *ibid.*, xxxiii, p. 311, 1902.

<sup>5</sup> Schrauth and Schoeller: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 2812, 1912.

<sup>6</sup> Dimroth: *ibid.*, xxxv, p. 2039, 1902.

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*4-p-Dimethylaminobenzeneazophenylmercuric acetate.* 3.5 grams of *p*-aminophenylmercuric acetate were suspended in 50 cc. of water, 20 cc. of glacial acetic acid added, warmed to facilitate solution, cooled with ice, and diazotized, drop by drop, with an aqueous solution of 0.7 gram of sodium nitrite. To the orange-brown solution obtained after the removal of a small amount of what was probably the diazoamino compound, a solution of 1.2 grams of dimethylaniline in acetic acid was added, causing rapid formation of the dye. After several hours this was filtered off, washed with water and alcohol, and recrystallized from amyl alcohol. Yield: 1.2 grams. The substance forms minute, brick-red crystals with a violet reflex, melts at 215° (uncorrected), and, in acetic acid solution, dyes silk a bright yellow color. It is difficultly soluble in the usual solvents, with the exception of acetic acid, in which it dissolves with a bright red color. In concentrated sulphuric acid the color is red-brown with a slight green fluorescence, while dilute nitric and hydrochloric acids form dark colored, very difficultly soluble salts. For analysis and melting point determination the substance was dried to constant weight at the temperature of boiling alcohol *in vacuo* over sulphuric acid.

0.3184 gm. of substance gave 0.1553 gm. HgS. 0.1559 gm. of substance gave 12.50 cc. moist N (755 mm. and 28.2°).

Calculated for  $C_{16}H_{17}O_2N_2Hg$ : Hg = 41.47 per cent. N = 8.69 per cent.

Found: Hg = 42.06 per cent. N = 8.69 per cent.

*4-p-Diethylaminobenzeneazophenylmercuric acetate.* This was prepared similarly. Yield: 1.5 grams. The substance was obtained by crystallization from absolute alcohol as glistening, orange-brown platelets, soluble in sulphuric acid with an orange color, in acetic acid with an orange-red color, and, in general, somewhat more soluble than the preceding compound. The portion dried to constant weight *in vacuo* at room temperature over sulphuric acid reddened above 120°, melted at 154.5–6° (corrected), and analyzed as follows:

0.2545 gm. of substance gave 0.1151 gm. HgS. 0.1572 gm. gave 11.3 cc. moist N (767 mm. and 23.5°).

Calculated for  $C_{18}H_{21}O_2N_2Hg$ : Hg = 38.19 per cent. N = 8.21 per cent.

Found: Hg = 38.99 per cent. N = 8.12 per cent.

*4-p-Oxybenzeneazophenylmercuric acetate.* This was obtained by diazotizing *p*-aminophenylmercuric acetate as above and pour-

ing the diazo solution, with cooling and stirring, into a solution of phenol (2 mols.) in enough 10 per cent aqueous sodium hydroxide to maintain an alkaline reaction at the end. After standing over night the dye was salted out by means of sodium acetate, freed from impurities by solution in dilute aqueous sodium hydroxide, precipitated by acetic acid, and recrystallized from this solvent, forming orange crystals melting at 218–9° (corrected). In alkaline solution it dyes silk a light yellow shade. For analysis, the compound was dried to constant weight at the temperature of boiling alcohol *in vacuo* over sulphuric acid. The product is isomeric with that obtained by Dimroth from benzenediazonium chloride and *o,p*-oxyphenyldimercuric diacetate.<sup>7</sup>

0.1894 gm. of substance gave 0.0964 gm. HgS. 0.1528 gm. gave 9.0 cc. moist N (762 mm. and 23.3°).

Calculated for  $C_{14}H_{12}O_2N_2Hg$ : Hg = 43.92 per cent. N = 6.14 per cent.  
Found: Hg = 43.88 per cent. N = 6.61 per cent.

*4-o, p-Dioxybenzeneazophenylmercuric acetate.* This was formed by diazotizing 3.5 grams of *p*-aminophenylmercuric acetate and pouring the diazo solution into a well cooled solution of 1.5 grams of resorcin in 200 cc. of 10 per cent aqueous sodium hydroxide. After standing for one hour the deep orange-brown solution was filtered, precipitated with acetic acid, and boiled for about one hour with 50 per cent acetic acid in order to cause complete re-conversion into the acetate. In this way 1.4 grams were obtained. Owing to its insolubility the substance could not be recrystallized. It forms a dark brown powder, soluble in sulphuric acid and in dilute alkali with an orange-brown color, the latter solution dyeing silk an orange shade. The portion dried to constant weight for analysis at 100° *in vacuo* over sulphuric acid darkened above 160°, gradually softened, and decomposed from 190° to 195°.

0.2668 gm. of substance gave 0.1337 gm. HgS. 0.1504 gm. gave 8.0 cc. moist N (774 mm. and 17.5°).

Calculated for  $C_{14}H_{12}O_4N_2Hg$ : Hg = 42.43 per cent. N = 5.93 per cent.  
Found: Hg = 43.21 per cent. N = 6.24 per cent.

*1-Amino-2-[p-naphthaleneazophenylmercuric acetate]-5-sulphonic acid.* This was prepared by adding slowly, with stirring, the fil-

<sup>7</sup> Dimroth: *ibid.*, xxxv, p. 2863, 1902.

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tered diazo solution from 3.5 grams of *p*-aminophenylmercuric acetate to a suspension obtained by dissolving 2.5 grams of  $\alpha$ -naphthylamine-5-sulphonic acid in 30 cc. of hot, saturated sodium acetate solution and cooling rapidly. The dye separated almost immediately. After standing over night the mixture was filtered and the precipitate dissolved in very dilute aqueous sodium hydroxide, the sodium salt being very insoluble in the presence of sodium ion. The dye was again salted out with solid sodium acetate, dissolved in water, filtered, precipitated by acetic acid, and filtered as well as possible with the aid of suction. The pasty mass thus obtained was dried over sulphuric acid *in vacuo*, pulverized, washed several times with water, then with a little alcohol, and dried again to constant weight at room temperature *in vacuo* over sulphuric acid. Yield: 1.9 grams. Isolated in this way, the substance forms a glistening, brown-black, micro-crystalline powder, practically insoluble in dilute hydrochloric acid, soluble in sulphuric acid with an orange-red color, and soluble in dilute aqueous sodium hydroxide with a deep maroon color, the solution dyeing silk an orange shade. The compound decomposes at about 280°.

0.3071 gm. of substance gave 0.1215 gm. HgS.

Calculated for  $C_{11}H_{10}O_4N_2SHg$ : Hg = 34.24 per cent.

Found: Hg = 34.09 per cent.

*4-o-Oxybenzylideneaminophenylmercuric acetate.* A suspension of 3.5 grams of finely powdered *p*-aminophenylmercuric acetate in absolute alcohol containing 1.5 grams of salicylic aldehyde was heated to boiling. Condensation took place almost immediately, and frequent stirring was necessary to break up the thick mass formed. After one-half hour the mixture was cooled, filtered, and the substance recrystallized from benzene. From this solvent it separated as deep yellow micro-crystals, darkening, when rapidly heated, above 140° and melting with decomposition at about 185°. Yield: 1.7 grams. The substance is soluble in dilute aqueous sodium hydroxide, chloroform, and acetic acid, and is hydrolyzed with difficulty on boiling with dilute hydrochloric acid. For analysis, the compound was dried to constant weight at the temperature of boiling alcohol *in vacuo* over sulphuric acid.

0.2596 gm. of substance gave 0.1328 gm. HgS. 0.2286 gm. gave 6.35 cc. moist N (757 mm. and 24.7°).

Calculated for  $C_{11}H_{13}O_2NHg$ : Hg = 44.02 per cent. N = 3.07 per cent.  
Found: Hg = 44.11 per cent. N = 3.08 per cent.

The above azomethine, as well as a number of others which we have prepared, was accompanied by by-products the nature of which we are investigating.

## 2. Action of nitrous acid on *p*-methylaminophenylmercuric acetate.

*p*-Methylnitrosoaminophenylmercuric acetate. 3.6 grams of crude *p*-methylaminophenylmercuric acetate were suspended in a little water, acetic acid was added until maximum solubility was reached, the solution filtered from the insoluble portion, cooled with ice, and treated with a solution of 0.7 gram of sodium nitrite in a little water. On stirring, 0.9 gram of nitroso compound crystallized out, to which was added 0.2 gram that separated from the diluted mother liquor on standing over night. After two recrystallizations from absolute alcohol there remained 0.9 gram of pale drab-colored hexagonal platelets, melting at 183–4° (corrected) with decomposition. The substance dissolves readily in acetone and chloroform, and gives a slowly developing olive color with a solution of phenol in sulphuric acid. For analysis and melting point determination the compound was dried to constant weight at room temperature *in vacuo* over sulphuric acid.

0.2616 gm. of substance gave 0.1567 gm. HgS. 0.1745 gm. gave 10.95 cc. moist N (761 mm. and 23°).

Calculated for  $C_8H_{10}O_2N_2Hg$ : Hg = 50.82 per cent. N = 7.10 per cent.  
Found: Hg = 51.64 per cent. N = 7.05 per cent.

## 3. 3-Methyl-4-aminophenylmercuric derivatives.

3-Methyl-4-aminophenylmercuric acetate was prepared by Schrauth and Schoeller,<sup>8</sup> who, however, isolated the substance as the chloride. We find that the acetate, when prepared by rapidly adding an alcoholic solution of three molecular equivalents of *o*-toluidine to a solution of one equivalent of mercuric acetate in 50 per cent alcohol, contains some of the dimercuridiacetate,

<sup>8</sup> Schrauth and Schoeller: *loc. cit.*



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but is readily purified, although with considerable loss, by recrystallizing, first from 50 per cent alcohol, then from absolute alcohol. The acetate is easily soluble in hot water, difficultly in ethyl acetate. As it decomposed slowly on heating *in vacuo* at 100° over sulphuric acid, it was dried to constant weight at room temperature. Plunged into a bath at about 140°, the substance melts at 144–5° with preliminary softening.

0.2570 gm. of substance gave 0.1651 gm. HgS. Kjeldahl. 0.2198 gm. required 5.8 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_9H_{11}O_2NHg$ : Hg = 54.85 per cent. N = 3.83 per cent.  
Found: Hg = 55.39 per cent. N = 3.69 per cent.

**3-Methyl-4-p-oxybenzeneazophenylmercuric acetate.** 3.6 grams of 3-methyl-4-aminophenylmercuric acetate were dissolved in 50 cc. of water and 15 cc. of acetic acid with gentle heating, cooled below 10°, and slowly diazotized with a solution of 0.7 gram of sodium nitrite. The clear, orange-brown solution was slowly added, with stirring and cooling, to a solution of 2 grams of phenol in 200 cc. of 10 per cent sodium hydroxide solution containing a little ice. After standing until it no longer reacted with R-salt paper, the mixture was filtered to remove a small amount of insoluble matter and precipitated with carbon dioxide. After two crystallizations from 85 per cent alcohol containing a little acetic acid, the substance was dried to constant weight at room temperature *in vacuo* over sulphuric acid. When rapidly heated up to 200°, the compound melts at 204.5–5° (corrected), with preliminary sintering.

0.2170 gm. of substance gave 0.1077 gm. HgS. 0.1364 gm. gave 6.6 cc. moist N (772 mm. and 20.7°) when burned with lead chromate.

Calculated for  $C_{11}H_{14}O_2N_2Hg$ : Hg = 42.61 per cent. N = 5.95 per cent.  
Found: Hg = 42.79 per cent. N = 5.58 per cent.

## THE PREPARATION AND MELTING POINTS OF THE HIGHER ALIPHATIC HYDROCARBONS.

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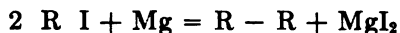
The higher hydrocarbons are of interest to the chemist not only for their practical and theoretical features, but also for their bearing on the identification of fatty acids. The number of carbon atoms in the higher fatty acids can not be recognized by direct analysis. The differences in their carbon and hydrogen contents is practically within the limits of error of the analytical methods. Also, the molecular weight estimation alone was frequently found insufficient to permit a definite opinion as to the size of the molecule of a fatty acid.

Under such circumstances one naturally formed conclusions on the basis of the physical constants of the acid and of its derivatives. And in this connection it was often found convenient to reduce a fatty acid to the corresponding hydrocarbon, which could be then compared with one of the known hydrocarbons.

In the course of our work on the structure of the naturally occurring fatty acids, of as yet undetermined structure, we felt that a revision of the accepted melting points of the higher hydrocarbons was much desired for two principal reasons. First, the older methods of preparation of some of them did not offer sufficient assurance of their purity: they were obtained either by vigorous reduction directly from fatty acids or ketones (Krafft), or by fractionation of naturally occurring mixtures (Mabery). Second, a long series of hydrocarbons was seldom prepared by the same worker, and hence the procedure in determining the melting points might have suffered from a lack of uniformity. And yet the rate of heating undoubtedly determines the temperature of melting of substances which melt at a comparatively low temperature.

The improvement<sup>1</sup> in the methods of reduction of the esters of fatty acids has made easy the access to the corresponding alcohols. In their turn these are transformed readily into the corresponding iodides. Finally, the iodides can be reduced very easily by means of zinc and hydrochloric acid to the corresponding hydrocarbons. Thus the hydrocarbon is arrived at through a greater number of operations than was required by the old hydriodic acid process, but every step in the newer process requires comparatively mild treatment.

Tetracosane and the higher paraffins were prepared by the action of magnesium in dry ether on the iodides containing half the number of carbon atoms in their molecule, according to the well known reaction:



In determining the melting points great care was taken in guarding uniformity of heating. The observations were carried out in a sulphuric acid bath provided with a stirring arrangement, and the rate of heating was six to seven seconds per degree for the terminal 15°. The results of the observation are recorded in the following table.

TABLE I.

HYDROCARBONS	MELTING POINTS GIVEN BY MEYER-JACOBSON	OTHER MELTING POINTS	LEVENE, WEST, AND VAN DER SCHEER
	°C	°C	°C
C <sub>16</sub> H <sub>34</sub> .....	18	19-20	20
C <sub>18</sub> H <sub>38</sub> .....	28		28
C <sub>20</sub> H <sub>42</sub> .....	37		38
C <sub>22</sub> H <sub>46</sub> .....	44		47
C <sub>24</sub> H <sub>50</sub> .....	51		54
C <sub>26</sub> H <sub>54</sub> .....	58		59-60
C <sub>28</sub> H <sub>58</sub> .....	60		64-65
C <sub>30</sub> H <sub>62</sub> .....	—	65.2-65.5	69-70
C <sub>32</sub> H <sub>66</sub> .....	70	70.5	74-74.5
C <sub>34</sub> H <sub>70</sub> .....	—	73.2	76-76.5
C <sub>36</sub> H <sub>74</sub> .....	—	76, 76.5	78.5

We have also determined the melting points of the higher hydrocarbons obtained by the reduction of lignoceric alcohol (from lignoceric acid), of ceryl alcohol, and of melissyl (myricyl) alcohol.

<sup>1</sup> L. Bouveault and G. Blanc: *Compt. rend. Acad. d. sc.*, cxxxvi, p. 1676, 1903; cxxxvii, p. 60, 1903.

The significance of these melting points will be discussed later when more information on the structure of their mother substances has been obtained.

## EXPERIMENTAL.

*Hexadecane, C<sub>16</sub>H<sub>34</sub>*

A sample of hexadecane was first prepared by reducing Kahlbaum's cetyl iodide. Suspended in glacial acetic acid, it was treated with a large amount of zinc dust and warmed on the water bath for two days; dry hydrochloric was occasionally passed into the reaction mixture to hasten the reaction. This preparation, when cooled, filtered, and dried in ethereal solution, boiled over a wide range of temperature, and when fractionated, gave products which melted at from 16° to 19°.

We then fractionated the cetyl iodide (two different preparations) and found, to our surprise, that this showed a similar range in its boiling point. An attempt to separate this into fractions and to reduce the various fractions did not give a satisfactory product.

Finally, cetyl alcohol was prepared by the reduction of ethyl palmitate with sodium and absolute ethyl alcohol<sup>1</sup> and this converted into the iodide by heating with molecular proportions of red phosphorus and iodine. Such a sample boiled constantly at 152° under 0.6 mm. pressure.

0.1665 gm. of substance gave 0.1110 gm. AgI (Carius).

	Calculated for C <sub>16</sub> H <sub>34</sub> I:	Found:
I.....	36.08	36.04

Reduced as above a hydrocarbon was obtained which boiled at 110° and 1 mm., and melted at 20°. The melting point given in the literature is 17°,<sup>2</sup> 18°,<sup>3</sup> and 19–20°.<sup>4</sup>

0.0870 gm. of substance gave 0.2711 gm. CO<sub>2</sub> and 0.1170 gm. H<sub>2</sub>O.

	Calculated for C <sub>16</sub> H <sub>34</sub> :	Found:
C.....	84.85	85.01
H.....	15.15	15.01

<sup>1</sup> E. Eichler: *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1882, 1879.

<sup>2</sup> F. Krafft: *ibid.*, xv, p. 1702, 1882.

<sup>4</sup> B. Lachowicz: *Ann. d. Chem.*, cexx, p. 181, 1883.

*Octadecane, C<sub>18</sub>H<sub>38</sub>*

In the first attempts to prepare octadecane we tried to reduce  $\alpha$ -hydroxystearic acid with hydriodic acid and red phosphorus, using the procedure previously described for the reduction of ceribronic acid.<sup>5</sup> The hydrocarbon was undoubtedly formed in the reaction, but it was almost impossible to obtain it in a pure state.

We then thought to reduce octadecyl alcohol. The alcohol, obtained by the reduction of ethyl stearate, melted at 58.5° and boiled at 210° under 15 mm. pressure. It is best recrystallized from dry acetone. 5 grams of this alcohol, 2 grams of red phosphorus, and 15 cc. of hydriodic acid (density 1.96) were heated in a sealed tube for eight hours at 140°. The product after removal of the excess acid with water and of the iodine by treatment with sodium thiosulphate in ether solution, boiled at 224° under 2 mm. pressure. Analysis showed it to be a mixture of a little hydrocarbon with much octadecyl iodide.

0.1734 gm. of substance gave 0.1012 gm. AgI (Carius).

	Calculated for C <sub>18</sub> H <sub>37</sub> I:	Found:
I.....	33.41	31.54

The pure iodide was obtained by heating 25 grams of alcohol, 12 grams of iodine, and 2 grams of red phosphorus for one hour at 180°. Recrystallized from acetone it melted at 34° and boiled at 169–170° at 0.5 mm. pressure.

0.1868 gm. of substance gave 0.1140 gm. AgI (Carius).

	Calculated	Found:
I.....	33.40	33.00

*Octadecane* was prepared from the impure or pure iodide by reducing with zinc dust and hydrochloric acid in glacial acetic solution. The solid which separated out on cooling was filtered off and the mother liquor concentrated to dryness. Both products were taken up in ether, the solution was washed with water and, after drying, distilled in vacuum. The hydrocarbon boiled at 177° under 15 mm. pressure and melted at 28°. This is the melting point reported by Krafft,<sup>6</sup> who obtained it by the action

<sup>5</sup> P. A. Levene and C. J. West: this *Journal*, xiv, p. 257, 1913.

<sup>6</sup> Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 1703, 1882; xix, p. 2221, 1886.

of sodium upon nonyl iodide and by reducing stearic acid with hydriodic acid at 240°.

0.1200 gm. of substance gave 0.3791 gm. CO<sub>2</sub> and 0.1619 gm. H<sub>2</sub>O.

	Calculated for C <sub>20</sub> H <sub>42</sub> :	Found:
C.....	84.94	84.80
H.....	15.06	15.10

*Eicosane, C<sub>20</sub>H<sub>42</sub>*

*Preparation of arachidic acid.* After unsuccessful attempts to isolate analytically pure arachidic acid from the mixture of fatty acids obtained by the hydrolysis of peanut oil,<sup>7</sup> we prepared it by the fusion of erucic acid with alkali.<sup>8</sup> Best results were obtained by fusion of lots of 10 grams.

10 grams of erucic acid and 5 grams of solid potassium hydroxide were heated in a nickel dish, with or without the addition of a few drops of water until the soap was formed. 15 grams of potassium hydroxide were then added and the heating was carefully continued until the mass was molten. When further heated the mass again becomes solid, darkens very much, and finally chars and even takes fire. The reaction takes place apparently at this final stage of the heating. If the heating is stopped when the mass is a dark brown and the acid isolated, a product is obtained which melts at 65–66°, and is not changed by recrystallization from alcohol or acetone. This was not analyzed, but it is probably brassic acid, an isomer of erucic acid. The black reaction product was dissolved in water, the solution acidified and warmed until the fatty acids were molten, then cooled and filtered. The fatty acids were dissolved in a little alcohol, the solution was filtered and cooled. After two or three recrystallizations a pure arachidic acid was obtained, melting at 77°. In order to insure the complete removal of any unsaturated acids, the product from the

<sup>7</sup> Compare H. Meyer, L. Brod, and W. Soyka: *Monatsh. f. Chem.*, xxxiv, p. 1113, 1913. We have been able, however, to obtain fair yields of pure lignoceric acid, melting at 80–81°, by fractionating the more insoluble fractions.

<sup>8</sup> R. Willstätter, E. W. Mayer, and E. Hüni (*Ann. d. Chem.*, cccxxviii, p. 73, 1911) mention that they have used this method of preparation, but give no details. Because of the trouble of finding the best conditions for this fusion we have given rather full details.

first crystallization out of alcohol was changed into the lead salt, this twice recrystallized out of toluene at room temperature and the lead salt decomposed as usual with hydrogen sulphide.

Twice recrystallized out of toluene at room temperature the acid melted sharply at 77°.

1.000 gm. of acid, dissolved in a mixture of benzene and methyl alcohol required 31.9 cc.  $\frac{N}{10}$  HCl for neutralization.

	Calculated for $C_{20}H_{40}O_2$ :	Found:
Mol. wt.....	312.3	313.1

**Eicosyl alcohol.** Ethyl arachidate was reduced with sodium and amyl alcohol and the mixture of soap and alcohol worked up in the usual manner. 90 grams of ester gave 35 grams of alcohol, which boiled at 210° and 0.3 mm. pressure and melted at 66–67°, after recrystallization from dry acetone. The melting point given by Haller,<sup>9</sup> who first prepared it in the same way, is 71°, while Willstätter<sup>10</sup> gives 63–64°.

**Eicosyl iodide.** 15 grams of eicosyl alcohol, 11.5 grams of iodine, and 1.5 grams of red phosphorus were heated in a metal bath at 180° for one hour and twenty-five minutes. The reaction product was taken up in ether, purified with sodium thiosulphate, and distilled. It boiled at 192° under 0.5 mm. pressure and melted at 42°, after recrystallization from dry acetone.

0.1847 gm. of substance gave 0.1048 gm. AgI (Carius).

	Calculated for $C_{20}H_{41}I$ :	Found:
I.....	31.01	30.67

**Eicosane** was prepared in the usual manner by reducing eicosyl iodide with zinc dust and hydrochloric acid. It boils at 148° at 0.6 mm. pressure and melts at 38°. Krafft<sup>11</sup> gives the melting point as 36.7°.

<sup>9</sup> A. Haller: *Compt. rend. Acad. d. sc.*, cxliv, p. 594, 1907.

<sup>10</sup> Willstätter, Mayer, and Hüni: *loc. cit.*

<sup>11</sup> Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 1717, 1882; xix, p. 2220, 1886.

0.1037 gm. of substance gave 0.3228 gm. CO<sub>2</sub> and 0.1406 gm. H<sub>2</sub>O.

	Calculated for C <sub>22</sub> H <sub>46</sub> :	Found:
C.....	85.01	84.92
H.....	15.00	15.14

*Docosane, C<sub>22</sub>H<sub>46</sub>*

We first attempted to prepare the necessary docosyl alcohol by the reduction of erucyl alcohol. This was obtained by the reduction of ethyl erucate with sodium and amyl alcohol. Difficulty was found in the extraction of the mixture of soap and alcohol with ether or petroleum ether, since some of the soap always went into solution and did not separate again upon drying the solution at 0°. This soap prevented further purification because in the distillation it caused violent foaming. The best results were obtained by the use of dry acetone. The erucyl alcohol thus obtained boiled at 199–200° at 0.2 mm. pressure and melted at 31–32°. Recrystallized from a little acetone, it melted at 33–34°, while Willstätter gives 34–35° after recrystallization from methyl alcohol at –30°.

Difficulty was again encountered in an attempt to reduce the alcohol with hydrogen, using colloidal palladium as a catalyzer, little or no hydrogen being absorbed by a solution of the alcohol in ethyl alcohol, amyl alcohol, ether, or acetone. This may be due to the presence of slight traces of ash. In methyl alcoholic solution about 50 per cent was reduced, though the method was hardly suitable for preparative purposes. The docosyl alcohol was separated from unchanged erucyl alcohol by crystallization from acetone and from chloroform. It melted at 73–74°. Willstätter gives 71–71.5°; Meyer, 71°.

For the preparation of a larger quantity of docosyl alcohol we reduced erucic acid to behenic acid by the palladium hydrogen method; the acid melted at 84° after one recrystallization from acetone. We never had the difficulty mentioned by Meyer of obtaining a product with a low melting point. This was esterified and reduced with sodium and amyl alcohol. Recrystallized out of acetone and then from chloroform it melted at 73–74°.



0.1050 gm. of substance gave 0.3111 gm.  $\text{CO}_2$  and 0.1342 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{22}\text{H}_{46}\text{O}$ :	Found:
C.....	80.88	80.80
H.....	14.22	14.30

Docosyl iodide was prepared by heating 15 grams of docosyl alcohol, 6 grams of iodine, and 2 grams of red phosphorus for one hour at  $180^\circ$ . Recrystallized from absolute ether it melted at  $49^\circ$ . Meyer, Brod, and Soyka give  $46^\circ$ .

0.1934 gm. of substance gave 0.1028 gm.  $\text{AgI}$  (Carius).

	Calculated for $\text{C}_{22}\text{H}_{46}\text{I}$ :	Found:
I.....	29.1	28.7

*Docosane*. Docosyl iodide was converted into the hydrocarbon by the usual method. The product was distilled in vacuum and then recrystallized several times from dry acetone and finally from absolute ether. *Docosane* melts at  $47^\circ$ . Krafft<sup>12</sup> gives the melting point as  $44.4^\circ$ .

0.0990 gm. of substance gave 0.1300 gm.  $\text{H}_2\text{O}$  and 0.3086 gm.  $\text{CO}_2$ .

	Calculated for $\text{C}_{22}\text{H}_{46}$ :	Found:
C.....	85.05	85.02
H.....	14.94	14.70

#### *Tetracosane*, $\text{C}_{24}\text{H}_{50}$

The preparation and properties of this hydrocarbon, as well as the isomeric hydrocarbon obtained from lignoceric acid have been described in an earlier article.<sup>13</sup> The normal hydrocarbon melts at  $54^\circ$ , the iso hydrocarbon at  $51^\circ$ . The name *lignocerane* is suggested for this iso compound until the constitution of the lignoceric acid is definitely established.

#### *Hexacosane*, $\text{C}_{26}\text{H}_{54}$

Tridecyl alcohol<sup>14</sup> was obtained by reducing ethyl tridecylate with sodium and absolute ethyl alcohol. It boils at  $117^\circ$  at 0.5 mm. pressure.

*Tridecyl iodide* was prepared by heating 25 grams of alcohol, 16.5 grams of iodine, and 3 grams of red phosphorus for one hour

<sup>12</sup> Krafft: *ibid.*, xv, p. 1718, 1882.

<sup>13</sup> Levene and West: this *Journal*, xviii, p. 477, 1914.

<sup>14</sup> J. Blau: *Monatsh. f. Chem.*, xxvi, p. 103, 1905.

at 180°. Purified with sodium thiosulphate it was obtained as a colorless oil, boiling at 117° and 0.5 mm. pressure.

0.1794 gm. of substance gave 0.1350 gm. AgI (Carius).

	Calculated for $C_{18}H_{38}I$ :	Found:
I.....	40.39	40.69

*Hexacosane* was prepared by the action of 0.9 gram of magnesium upon 10 grams of tridecyl iodide in 150 cc. of absolute ether (freshly dried over sodium). The reaction proceeds slowly and is complete after boiling gently for five or six hours. Upon cooling the reaction mixture the hydrocarbon separates out. The product was brought into solution by adding more ether and warming, the residue of the magnesium filtered off, the solution treated with dilute hydrochloric acid, washed free of acid with water, and the hydrocarbon crystallized out of the concentrated solution. This was distilled in vacuum to remove traces of ash, the substance boiling at 199° and 0.4 mm. pressure. Twice recrystallized out of ether it formed glistening scales which melted at 59–60°. Mabery<sup>15</sup> isolated a hydrocarbon from petroleum having this composition and melting at 58°.

0.0944 gm. of substance gave 0.2946 gm. CO<sub>2</sub> and 0.1234 gm. H<sub>2</sub>O.

	Calculated for $C_{26}H_{54}$ :	Found:
C.....	85.17	85.12
H.....	14.83	14.63

#### *Octacosane*, $C_{28}H_{58}$

Tetradecyl iodide was prepared according to Majima and Nakamura.<sup>16</sup> It boiled at 128° under a pressure of 0.5 mm.

0.1750 gm. of substance gave 0.1262 gm. AgI (Carius).

	Calculated for $C_{14}H_{30}I$ :	Found:
I.....	39.17	38.99

The action of magnesium upon an ether solution of tetradecyl iodide, gave *octacosane*, glistening leaflets from absolute ether, boiling at 224° under 1.1 mm. pressure, and melting at 64–65°. Mabery<sup>15</sup> gives 60° as the melting point of the product isolated from petroleum.

<sup>15</sup> C. F. Mabery: *Am. Chem. Jour.*, xxviii, pp. 193, 195, 1902.

<sup>16</sup> R. Majima and I. Nakamura: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 4094, 1913.

## 530 Higher Aliphatic Hydrocarbons

0.0968 gm. of substance gave 0.3026 gm. CO<sub>2</sub> and 0.1266 gm. H<sub>2</sub>O.

	Calculated for C <sub>25</sub> H <sub>52</sub> :	Found:
C.....	85.17	85.25
H.....	14.82	14.63

### *triacontane*, C<sub>30</sub>H<sub>62</sub>

This hydrocarbon has recently been prepared by Gascard,<sup>17</sup> through the action of sodium upon pentadecyl iodide in boiling xylene for ten hours.

Pentadecyl iodide, prepared from the alcohol with iodine and red phosphorus, boiled at 138–139° under 0.5 mm. pressure.

0.1763 gm. of substance gave 0.1220 gm. AgI (Carius).

	Calculated for C <sub>15</sub> H <sub>31</sub> I:	Found:
I.....	37.78	37.43

Magnesium, acting upon this iodide in ethereal solution, forms *triacontane*, which separated from the reaction mixture as glistening scales. These boiled at 235° under a pressure of 1 mm. and melted at 69–70°. Gascard gives 65.2–65.5°.

0.1015 gm. of substance gave 0.3169 gm. CO<sub>2</sub> and 0.1331 gm. H<sub>2</sub>O.

	Calculated for C <sub>30</sub> H <sub>62</sub> :	Found:
C.....	85.21	85.22
H.....	14.79	14.68

### *Dotriacontane*, C<sub>32</sub>H<sub>66</sub>

Besides the older work on this hydrocarbon, it has recently been obtained by Ruttan<sup>18</sup> as a by-product in the preparation of margaric acid by the action of magnesium upon cetyl iodide, followed by the action of carbon dioxide, and by Gascard,<sup>19</sup> who prepared it by the action of sodium amalgam upon dotriacontanyl iodide (the dotriacontanol was isolated as an ester from gum lac). Both authors give the melting point as 70.5°.

The hydrocarbon has been obtained in a yield of 70 to 80 per cent by the action of magnesium upon cetyl iodide in absolute

<sup>17</sup> A. Gascard: *Compt. rend. Acad. d. sc.*, cliii, p. 1484, 1911.

<sup>18</sup> R. F. Ruttan: *VIIIth International Congress of Applied Chemistry*, xxv, p. 435, 1912; *Chem. Abstr.*, vii, p. 2190, 1913.

<sup>19</sup> Gascard: *Compt. rend. Acad. d. sc.*, clix, p. 258, 1914.

ether (moisture was not as rigorously excluded as in the case of Ruttan). Recrystallized from ether *dotriacontane* forms large lustrous plates or scales, boiling at 245° at 1.5 mm. pressure and melting at 74–75°.

0.1030 gm. of substance gave 0.3218 gm. CO<sub>2</sub> and 0.1358 gm. H<sub>2</sub>O.

	Calculated for C <sub>32</sub> H <sub>66</sub> :	Found:
C.....	85.24	85.21
H.....	14.76	14.76

*Tetratriacontane*, C<sub>34</sub>H<sub>70</sub>

Heptadecyl alcohol<sup>20</sup> was prepared by reducing ethyl margarate with sodium and amyl alcohol. Recrystallized from acetone it melted at 54°. The corresponding iodide boiled at 158–159° under 0.5 mm. pressure. 13 grams of iodide in 150 cc. of absolute ether, treated with 0.9 gram of magnesium, and boiled gently for six hours, gave *tetratriacontane*, glistening scales or leaflets from absolute ether, boiling at 255° and 1 mm. pressure and melting at 76–76.5°. Gascard gives the melting point as 73.2°.

0.1034 gm. of substance gave 0.3230 gm. CO<sub>2</sub> and 0.1356 gm. H<sub>2</sub>O.

	Calculated for C <sub>34</sub> H <sub>70</sub> :	Found:
C.....	85.26	85.21
H.....	14.74	14.76

*Hexatriacontane*, C<sub>36</sub>H<sub>74</sub>

*Hexatriacontane* is readily obtained by the action of magnesium upon an ethereal solution of octadecyl iodide. Twice recrystallized from petroleum ether (boiling point 60–70°) it forms lustrous leaflets and scales, boiling at 265° under 1 mm. pressure and melting at 78.5°. This hydrocarbon has previously been prepared by Gascard, through the action of sodium in a boiling xylene solution of octadecyl iodide for ten hours, and by Oskeck<sup>21</sup> who obtained it as a by-product in the preparation of nondecylic acid by the action of carbon dioxide upon octadecyl magnesium bromide. Contrary to Oskeck, who states that it is not volatile without decomposition in vacuum, we had no difficulty in distill-

<sup>20</sup> Gascard: *ibid.*, cliii, p. 1484, 1911.

<sup>21</sup> Oskeck: *Jour. Russ. Phys. Chem. Soc.*, xlvi, pp. 416–7; *Chem. Abstr.*, viii, p. 3185, 1914.

ing it in a vacuum of 1 mm. These authors give the melting point as 76° and 76.5°.

0.1012 gm. of substance gave 0.3160 gm. CO<sub>2</sub> and 0.1332 gm. H<sub>2</sub>O.

	Calculated for C <sub>26</sub> H <sub>54</sub> :	Found:
C.....	85.27	85.25
H.....	14.73	14.74

*Iso-hexacosane, cerane, C<sub>26</sub>H<sub>54</sub>*

Ceryl alcohol was obtained by the hydrolysis of Chinese wax, using the following procedure: 100 grams of wax were dissolved in 500 cc. of hot toluene, 750 cc. of normal alcoholic potassium hydroxide were added, and the mixture was heated to boiling for six hours; the toluene was removed by distillation with steam, the residue poured into two liters of saturated salt solution, and the precipitate of salts and alcohols filtered off and dried in the air. This mixture was then thoroughly extracted with gasoline boiling between 70° and 80°. The precipitate which forms from the gasoline is recrystallized from gasoline and then from acetone. The yield was about 25 grams, depending upon the thoroughness of the extraction. The alcohol melted at 80° and solidified at 79°.

0.1012 gm. of substance gave 0.3020 gm. CO<sub>2</sub> and 0.1252 gm. H<sub>2</sub>O.

	Calculated for C <sub>26</sub> H <sub>54</sub> O:	Found:
C.....	81.59	81.42
H.....	14.23	13.87

The acetate, prepared by boiling the alcohol with acetic anhydride for two hours and recrystallized from dry acetone, melts at 64–65°. Benedict and Ulzer<sup>22</sup> give 65°.

Ceryl iodide was obtained by heating 25 grams of alcohol, 5 grams of red phosphorus, and 9 grams of iodine for two hours at 180°. Recrystallized from acetone it melted at 56–57° and solidified at 56–55°. Ryan and Algar<sup>23</sup> give the melting point at 55–56°.

0.1708 gm. of substance gave 0.0800 gm. AgI (Carius).

	Calculated for C <sub>26</sub> H <sub>53</sub> I:	Found:
I.....	25.80	25.33

<sup>22</sup> R. Benedict and F. Ulzer: *Monatsh. f. Chem.*, ix, p. 581, 1888.

<sup>23</sup> H. Ryan and J. Algar: *Proc. Roy. Irish Acad.*, xxx, p. 97, 1913; *Chem. Abstr.*, vii, p. 3318, 1913.

The hydrocarbon, *cerane*, was obtained by reducing the iodide with zinc dust and hydrochloric acid in glacial acetic acid for two days. Recrystallized from absolute ether it forms glistening scales, which boil at 207° under 0.7 mm. pressure and melt at 61°. Since this is higher than the corresponding normal hexacosane, it is probable that ceryl alcohol and cerotic acid which are obtained by heating the alcohol with soda lime have a branched and not a normal carbon chain. Nafzger<sup>24</sup> obtained a hydrocarbon by the distillation of ethyl cerotate under ordinary pressure, to which he ascribed the formula  $C_{26}H_{54}$ . Since this melted at 44°, it is evident that either the hydrocarbon was very impure, or that it was some other decomposition product of the acid.

0.1026 gm. of substance gave 0.3202 gm.  $CO_2$  and 0.1346 gm.  $H_2O$ .

	Calculated for $C_{26}H_{54}$	Found:
C.....	85.15	85.12
H.....	14.85	14.68

*Iso-triacontane, melissane,  $C_{30}H_{62}$*

Melissyl alcohol was obtained by the hydrolysis of carnauba wax in practically the same way as described above for ceryl alcohol; the hydrolysis was carried out in alcoholic-xylene solution. The precipitate of alcohol obtained from the gasoline was recrystallized from a large volume of dry acetone. Melissyl alcohol thus prepared has a melting point of 87.5–88° and solidifies at 87°. The melting point of the alcohol has been variously given as 85° by Brodie<sup>25</sup> and Pieverling,<sup>26</sup> 85.5–85.7° by Stürcke,<sup>27</sup> by Schwalb,<sup>28</sup> and by Benedict and Ulzer,<sup>29</sup> and 88° by Gascard<sup>30</sup> and by Matthes and Sander.<sup>31</sup> In this way 55 to 60 grams of pure alcohol may be easily obtained from 200 grams of carnauba wax.

<sup>24</sup> F. Nafzger: *Ann. d. Chem.*, cxxiv, p. 236, 1884.

<sup>25</sup> B. C. Brodie: *ibid.*, lxxi, p. 147, 1849.

<sup>26</sup> L. von Pieverling: *ibid.*, clxxxiii, p. 346, 1876.

<sup>27</sup> H. Stürcke: *ibid.*, cxxiii, p. 292, 1884.

<sup>28</sup> F. Schwalb: *ibid.*, cxxxv, p. 126, 1886.

<sup>29</sup> Benedict and Ulzer: *loc. cit.*

<sup>30</sup> Gascard: *Jour. de Pharm. et de Chim.*, series 5, xxviii, p. 49, 1893.

<sup>31</sup> H. Matthes and H. Sander: *Arch. d. Pharm.*, cxxlvi, p. 168, 1908.

0.1014 gm. of substance gave 0.3050 gm.  $\text{CO}_2$  and 0.1274 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{20}\text{H}_{42}\text{O}$ :	Found:
C.....	82.10	82.08
H.....	14.25	14.08

The acetate was prepared by boiling 2 grams of alcohol with 25 cc. of acetic anhydride for four hours. Recrystallized from dry acetone it melted at  $74\text{--}75^\circ$ . Benedict and Ulzer give its melting point as  $70^\circ$ , Gascard<sup>32</sup> as  $73^\circ$ , and Matthes and Sander as  $75^\circ$ .

0.1016 gm. of substance gave 0.2972 gm.  $\text{CO}_2$  and 0.1208 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{20}\text{H}_{40}\text{O}_2$ :	Found:
C.....	79.93	79.93
H.....	13.42	13.29

The iodide was prepared from 25 grams of alcohol, 8 grams of iodine, and 5 grams of red phosphorus by the usual method. The cooled reaction product was extracted with dry acetone, filtered hot from the excess of phosphorus, and recrystallized from dry acetone. It melted at  $70\text{--}71^\circ$  and solidified at  $69.5^\circ$ . Pieverling gives  $69.5^\circ$  as its melting point.

0.1902 gm. of substance gave 0.0816 gm.  $\text{AgI}$  (Carius).

	Calculated for $\text{C}_{20}\text{H}_{41}\text{I}$ :	Found:
I.....	23.14	23.20

The hydrocarbon, *melissane*, was prepared by reducing the iodide with zinc and hydrochloric acid. Recrystallized from gasoline (boiling at  $70\text{--}80^\circ$ ), it formed glistening scales and leaflets, which melted at  $73\text{--}74^\circ$ , and distilled at  $222^\circ$  under 0.3 mm. pressure. This melting point would likewise indicate a branch carbon chain for melissyl alcohol and melissic acid.

0.0968 gm. of substance gave 0.3025 gm.  $\text{CO}_2$  and 0.1272 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{20}\text{H}_{42}$ :	Found:
C.....	85.21	85.22
H.....	14.79	14.70

<sup>32</sup> Gascard: *Bull. Soc. chim.*, series 3, xi, p. 186, 1894.

## ANIMAL CALORIMETRY.

NINTH PAPER.

### THE INFLUENCE OF MEAT INGESTION ON THE AMINO-ACID CONTENT OF BLOOD AND MUSCLE.

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New York City.)

(Received for publication, January 20, 1915.)

Experiments performed by Professor Lusk<sup>1</sup> showed that after giving glycocoll and alanine to a dog there was a very considerable increase in the heat production of the animal, an increase which usually reached its maximum during the second hour after the amino-acid had been given. During the earlier hours there was only a slight increase in the quantity of nitrogen eliminated in the urine. These observations, together with the then recently published work of Folin,<sup>2</sup> which showed that after introduction of glycocoll into the gut there was an early increase in the quantity of "non-protein nitrogen" in the blood and muscles of a cat without any preliminary accumulation of urea, led to the conclusion that amino-acids such as glycocoll and alanine acted as stimuli to the tissues, increasing their heat production. The work of Van Slyke<sup>3</sup> also showed that there could be no question as to the avidity with which muscle absorbs amino-acids from the blood.

If an inert store of amino-acids in the muscle were the real cause of the heightened heat production, one would expect to find a decided increase in the amino-acid content of muscle after the administration of meat in large quantity. The present work was intended to solve this problem. It was completed before the publication of results by Van Slyke,<sup>4</sup> which led to similar conclusions.

<sup>1</sup> Lusk: this *Journal*, xiii, p. 155, 1912-13.

<sup>2</sup> O. Folin and W. Denis: *ibid.*, xii, p. 141, 1912.

<sup>3</sup> D. D. Van Slyke and G. M. Meyer: *ibid.*, xvi, p. 197, 1913-14.

<sup>4</sup> Van Slyke and Meyer: *loc. cit.*, p. 231.



The analytical methods outlined by Folin and Denis<sup>5</sup> were strictly followed, and analyses were made in duplicate.

The dogs were all maintained upon the "standard diet"<sup>6</sup> used habitually in this laboratory for animals of their weight. The blood was drawn from the carotid or femoral arteries either under cocaine or ether anesthesia, except in Experiment 2a, when it was withdrawn from an ear vein. The animal was killed by a quick blow on the head.

The following table shows analyses of blood and muscle after dogs had fasted for a short period, and comparative analyses of blood and muscle following the ingestion of 1000 grams of meat.

Although the experiments show wide variations in the individual dogs yet certain broad deductions are permissible. It is evident that *although the ingestion of 1000 grams of meat may increase the amount of amino-acids (as expressed by "residual N") and of urea in the circulating blood, yet in the muscles the quantity of amino-acids may show no positive increase though the urea content of the muscles rises.* These results show that if amino-acids from meat are absorbed by the muscle they are either immediately destroyed by the muscle or they are synthesized into new protein, and that they are not retained by the muscle to be gradually destroyed, since amino-acids do not accumulate in muscle following meat ingestion. It appears from this that the conclusion that the specific dynamic action of protein is due to the mass action of certain amino-acids acting in increased concentration on protoplasm is incorrect, and that the cause must be sought in the metabolic process involved in the transformation of these amino-acids.

The author acknowledges with gratitude the assistance of Miss Gertrude Fisher, who performed all the operative work described in this paper.

<sup>5</sup> Folin and Denis: *ibid.*, xi, p. 527, 1912.

<sup>6</sup> Lusk: *loc. cit.*, p. 185.

*The influence of meat ingestion upon the composition of 100 grams of blood and 100 grams of gastrocnemius muscle in the dog.*

NO. OF DOG	DATE	WEIGHT kgm.	TIME ON STANDARD DIET dys.	TIME FASTED hrs.	MEAT INGESTED gm.	TAKEN HRS. AFTER FOOD	TOTAL NON- PROTEIN N	UREA N	RESIDUAL N BY DIFFERENCE	VARIA TIONS
										IN RESID- UAL N FROM NORMAL AVERAGE
Blood										
I	Nov. 26, 1912	7.8	2	45			34	17	17	
II	Dec. 17, 1912	10.7	7	21			35	—	—	
IV	Jan. 30, 1913	7.8	14	25			32	15	17	
						Average	34	16	17	
IIa	Dec. 12, 1912	10.7	7	21	1000	3	66	20	46	+29
						6	54	34	20	+3
						9	71	43	28	+11
III	Jan. 3, 1913	10.2	17	25	1000	3	49	29	20	+3
VII	Feb. 7, 1913	10.2	30	25	1000	5	65	36	29	+12
VIII	Oct. 10, 1913	12.5	3	21	1000	5	54	35	19	+2
VI	Dec. 12, 1913	8.7	33	21	738	5	86	66	20	+3
						Average	64	38	27	
Muscle										
III	Feb. 1, 1913	10.2	17	25			180	66	113	
IV	Jan. 30, 1913	7.8	14	25			119	38	81	
V	May 5, 1913	7.0	8	25			190	47	145	
						Average	163	50	113	
I	Nov. 26, 1912	7.8	2	45	1000	3	152	37	115	+2
III	Jan. 3, 1913	10.2	17	25	1000	3	233	101	132	+19
VII	Mar. 7, 1913	10.2	30	25	1000	5	178	77	101	-12
VIII	Oct. 2, 1913	12.5	3	21	1000	5	166	56	117	-3
						Average	182	68	114	



# ANIMAL CALORIMETRY.

TENTH PAPER.

## THE RATE AT WHICH INGESTED GLYCOCOLL AND ALANINE ARE METABOLIZED.

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(Received for publication, January 20, 1915.)

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### I. INTRODUCTION.

Experiments published by Professor Lusk<sup>1</sup> two years ago showed that after the ingestion of glycoll or alanine by a dog there was a large increase in the heat production of the animal, which increase reached a maximum during the second or third hours after the acids had been taken and did not coincide with the maximum nitrogen output in the urine. Since Folin<sup>2</sup> had demonstrated that amino-acids absorbed from the intestine were largely deposited in the muscles, Lusk interpreted his results as indicating that the presence of these amino-acids in the muscles acted as stimuli to the protoplasm, causing an increase in metabolism. However, the investigations of Miss Wishart, described in the foregoing paper, failed to reveal any large accumulation of amino-acids in the muscles even after giving meat in quantity

<sup>1</sup> Lusk: this *Journal*, xiii, p. 155, 1912-13.

<sup>2</sup> O. Folin and W. Denis: *ibid.*, xii, p. 141, 1912.

sufficient nearly or quite to double the heat production of a dog. Since urea accumulates in the blood and tissues after the administration of meat or any of its cleavage products, the increase in the amount of urinary nitrogen might not be an index of the amino-acid metabolism during hourly periods. Former experiments by Reilly, Nolan, and Lusk<sup>3</sup> showed that after giving meat to completely phlorhizinized dogs the sugar derived from the metabolism of the meat was more rapidly eliminated than the urea, whose production was presumably almost coincident with that of the sugar.

It seemed probable, therefore, if 20 grams of glycocoll (from which 16 grams of glucose may arise) were given to a phlorhizinized dog and the urine was collected in hourly periods, that the elimination of extra sugar might be a better index of the rapidity of the metabolism of the ingested glycocoll than the increase in the nitrogen elimination. If this result was compared with the rate at which 16 grams of ingested glucose is eliminated, light might be thrown on the subject of the relative rapidity with which *exogenous* and *endogenous* glucose may be eliminated. Alanine likewise would lend itself to similar investigation.

## II. EXPERIMENTAL PROCEDURE.

After two days without food a dog was given 1 gram of phlorhizin suspended in 7 cc. of olive oil administered subcutaneously, and thereafter the same injection daily. On the second day following the phlorhizin injection the animal was immersed for several minutes in a cold bath at 10°C. and then placed for from four to six hours in the cold of a large ice box. This method rids the animal in large part of its glycogen reserve.<sup>4</sup> On the third day of phlorhization the ratio D: N<sup>5</sup> usually becomes constant, and interpretations may be drawn as regards the quantity of sugar which can be produced from a substance ingested by the dog.

<sup>3</sup> F. H. Reilly, F. W. Nolan, and G. Lusk: *Am. Jour. Physiol.*, i, p. 395, 1898.

<sup>4</sup> Lusk: *ibid.*, xxii, p. 163, 1908.

<sup>5</sup> The authors, Dakin and Woodyatt, write this as the G: N ratio on account of glucose being the true chemical name for the sugar in question. However, since the expression D: N has been in vogue for twenty years and is the accepted technical expression it is not deemed wise to change it at the present writing.

## III. EXPERIMENTAL PART.

A. *The effect of repeated phlorhization.*

Since the same dog was often phlorhized a second time after recovery from the first glycosuria, it was of importance to investigate the character of the second glycosuria. It is well known that a preliminary elimination of glucose from the blood and tissues of the body takes place as soon as phlorhizin begins to

TABLE I.

*The primary output of glucose after phlorhizin injection in dogs which had recovered from previous phlorhization.*

DOG	WEIGHT	DATE (1914)	HRS. SINCE LAST FOOD	TIME OF PHLORHIZIN INJECTION	PERIOD	GLU- COSE	NITRO- GEN	D:N	REMARKS
	kgm.					gm.	gm.		
I	13.2	Jan. 1		3.05 p.m.	3.05- 4.05 4.05- 5.10	1.52 2.52	0.244 0.239	6.23 10.57	First phlor- hization.
II	10.2	Feb. 14	40	9.48 a.m.	9.45-10.45 10.45-11.45 11.45-12.45	0.56 1.08 1.15	0.101 0.087 0.101	5.55 12.30 11.10	Second phlorhi- zination. Last previ- ous in- jection on Jan. 30.
III		Feb. 24	48	10.20 a.m.	10.20-11.20 11.20-12.20	1.01 1.51	0.170 0.134	5.92 11.26	Second phlorhi- zination. Last previ- ous in- jection on Feb. 4.
VI	12.8	May 4	20	1.00 p.m.	1.00- 3.00 3.00- 5.00	3.18 4.08	0.240 0.228	13.24 17.91	Third phlorhi- zination. Previous injection Apr. 25, 1913, and Mar. 20, 1914.

act upon the kidney. It was suggested that this phenomenon might be absent on administration of phlorhizin a second time. The preceding table indicates the fallacy of this hypothesis.

Dog VI mentioned in this table is Professor Lusk's Dog III as used in the calorimeter. The dog had been fed at 5.00 p.m. the previous evening, and the urine for two hours (10.46 to 12.46 p.m.) prior to the administration of phlorhizin contained 0.234 gram of nitrogen, as contrasted with 0.240 and 0.228 gram during two hour periods after phlorhization.

The results show that *the constant primary effect of phlorhizin is to cause the removal of sugar present in the organism without at first affecting the amount of protein metabolism.*

*The presence of the usual high D:N ratios shows that repetition of phlorhization has no effect upon the character of the initial stage of the glycosuria induced.*

#### *B. Two grams of Liebig's extract of beef.*

The ingestion of glyocoll and alanine solutions so frequently produce vomiting that it was found advantageous in some instances to administer them with the addition of 1 or 2 grams of Liebig's extract of beef. The nitrogen content of 2 grams of Liebig's extract is 0.156 gram and is eliminated in the normal animal within four hours after its ingestion.<sup>6</sup> The question to be settled was whether precursors of sugar existed in the material to such an extent as to modify the calculations of sugar arising from the amino-acids given with it. Therefore, 2 grams of Liebig's extract were given to a glycosuric dog on the sixth day after phlorhization and with the following results.

TABLE II.

*Dog VI.*

*Effect of administering 2 grams of Liebig's extract in 200 cc. of water to a phlorhizinized dog.*

DATE (1911)	PERIOD	GLUCOSE	NITROGEN	D:N	REMARKS
		gm.	gm.		
Mar. 16	<i>a.m.</i> 10.00-11.00	1.29	0.392	3.3	
	<i>a.m. p.m.</i> 11.00- 2.00	3.82	1.227	3.11	Dose at 11.00 a.m.

<sup>6</sup> Lusk: this *Journal*, xiii, p. 161, 1912-13.

In the hour prior to the administration of Liebig's extract, 1.29 grams of glucose were eliminated and during the three following hours the output of sugar was 1.27 grams per hour. The nitrogen elimination was only slightly increased. It is apparent that *2 grams of Liebig's extract are without perceptible influence upon the output of sugar in phlorhizin glycosuria.*

*C. Sixteen grams of glucose.*

Ringer and Frankel<sup>7</sup> gave 9 grams of glucose to a phlorhizinized dog and collected the urine in periods of two hours. In the successive periods 5.32, 2.34, 0.68, and 0.37 grams of extra glucose were eliminated, a total of 8.71 grams in eight hours.

Table III shows the results obtained after administering 16 grams of glucose.

The extra glucose may be variously calculated without greatly changing the result. The method chosen was to deduct 1.54 gm. of sugar eliminated hourly during the sixteen previous hours from the quantity of sugar eliminated hourly after the ingestion of sugar. This method indicates 16.15 grams of extra sugar in seven hours. If one bases the calculation on the assumption that the D:N ratio of the preceding hours should have been 3.65 and that the nitrogen metabolism held at the level of 0.373 gm. during the seven hours after glucose ingestion, the extra sugar eliminated would be

$$25.92 - (0.373 \times 7 \times 3.65) = 16.39 \text{ gm.}$$

It is evident from the table that the nitrogen elimination was largely increased after administering glucose, which fact may have been due to the diuresis following upon the ingestion of a large volume of water. There are, however, indications of a compensatory diminution of nitrogen elimination in the following hours. The average nitrogen output for the whole period of seven hours is 0.411 gm. per hour. Computing on this basis the extra glucose would amount to

$$25.92 - (0.411 \times 7 \times 3.65) = 15.53 \text{ gm.}$$

The three methods are essentially in agreement.

The hourly figures obtained on the following day show that the protein metabolism had risen to a higher level, so these figures could not be used.

Table I shows that, during the second two hour period, after the first injection of phlorhizin (in this same third phlorhization experiment on Dog VI), the sugar elimination was 2 grams per hour, or the level reached five hours after ingestion of 16 grams

<sup>7</sup> A. I. Ringer and E. M. Frankel: *ibid.*, xviii, p. 81, 1914.



TABLE III.

*Dog VI. Weight 11.5 kgm.**Elimination of glucose in hourly periods following the ingestion of 16 grams of glucose in 300 cc. of water.*

DATE (1914)	PERIOD	GLU- COSE	NITRO- GEN	D:N	EXTRA GLUCOSE		REMARKS
		gm.	gm.		gm.	per cent	
May 6-7	<i>p.m. a.m.</i> 5.00- 9.00	1.54	0.373	4.12			Urine con- taminat- ed with feces. Bladder urine at 9.00 a.m. D:N equals 3.68.
May 7	<i>a.m.</i> 10.00-11.00	3.91	0.465	8.40	2.37	14	At 10.15 a.m. 16 gm. glu- cose.
	11.00-12.00	6.65	0.576	11.55	5.11	32	
	<i>p.m.</i> 12.00- 1.00	5.21	0.391	13.32	3.67	22	
	1.00- 2.00	3.68	0.336	10.97	2.14	13	
	2.00- 3.00	2.67	0.364	7.34	2.14	13	
	3.00- 4.00	2.00	0.354	5.66	0.46	4	
	4.00- 5.00	1.80	0.389	4.65	0.26	2	
		25.92	*2.875		16.15		
May 8	<i>a.m. p.m.</i> 10.00- 1.00	1.84	0.517	3.54			

\* 0.411 gm. N is the average per hour.

of sugar. The kidney must have been readily able to eliminate this quantity of glucose.

The work of Fisher and Wishart<sup>3</sup> showed that there was a rapid though variable absorption of glucose when 50 grams were given to a normal dog, but that the absorption was completed during the fourth hour. On analyzing the results of Frankel and Ringer after giving 9 grams of glucose and comparing them with those after giving 16 grams, the following rate of extra sugar elimination may be calculated for two hour periods.

<sup>3</sup> G. Fisher and M. B. Wishart: *ibid.*, xiii, p. 53, 1912-13.

	9 GM. GLUCOSE		16 GM. GLUCOSE	
	Extra sugar	Per cent	Extra sugar	Per cent
1st 2 hrs.....	5.32	61	7.48	46
2d 2 hrs.....	2.34	27	5.82	35
3d 2 hrs.....	0.68	8	2.60	17
4th 2 hrs.....	0.37	4	0.73	2

When, therefore, 9 grams of glucose are ingested, 88 per cent may be eliminated during four hours, and when 16 grams are taken, 81 per cent may be so eliminated (94 per cent in five hours).

*Although there is certainly some lag in the excretion of sugar absorbed by the gut, yet its elimination by the phlorhizinized kidney appears to be almost as rapid as its absorption.*

TABLE IV.

*Dog V. Weight 16 kgm.*

*Elimination of glucose in hourly periods following the ingestion of 20 grams of glycocoll in 210 cc. of water.*

DATE (1914)	PERIOD	GLU- COSE	NITRO- GEN	D:N	EXTRA GLUCOSE		EXTRA NITROGEN		REMARKS
		gm.	gm.		gm.	per cent	gm.	per cent	
Apr. 22-23	p.m. a.m.								
	5.00- 8.00	2.43	0.719	3.40					
Apr. 23	a.m.								
	9.00-10.00	4.75	1.000	4.75	2.31	16	0.28	10	At 9.07 a.m.
	10.00-11.00	5.68	1.151	4.94	3.25	22	0.44	15	20 gm.
	11.00-12.00	5.61	1.270	4.44	3.19	22	0.55	18	glycocoll
	p.m.								(3.73 gm.
	12.00- 1.00	4.68	1.310	3.57	2.24	15	0.59	20	N).
	1.00- 2.00	4.01	1.107	3.63	1.58	11	0.39	13	
	2.00- 3.00	3.57	0.997	3.59	1.13	8	0.28	10	
	3.00- 4.00	2.88	0.921	3.12	0.43	3	0.20	7	
	4.00- 5.00	2.78	0.916	3.04	0.34	3	0.20	7	
Apr. 23-24					14.47		2.93		
	5.00-11.00	2.24	0.696	3.24					
	p.m. a.m.								
	11.00- 9.00	2.45	0.728	3.37					

*D. Twenty grams of glycocoll.*

Twenty grams of glycocoll were given to Dog V with the results shown in Table IV.

In this experiment the results are as clearly defined as one could wish, since the elimination of sugar and nitrogen and the

D:N ratios are essentially the same in the fore- as well as in the after-period. It appears that the protein metabolism of the period from 9.00 a.m. to 11.00 p.m., extending over fourteen hours, is the interval affected by the ingestion of glycocoll containing 3.73 grams of nitrogen. Deducting 3.73 grams of nitrogen derived from glycocoll from the total urinary nitrogen during these fourteen hours, which amounts to 12.84 grams, the difference is 9.11 grams (or 0.651 gram per hour). Multiplying this by the prevailing D:N ratio or 3.38, one finds that 30.79 grams of glucose could have arisen from the protein metabolism of the period. Since 47.42 grams of glucose were actually eliminated, it follows that the difference, or 16.63 grams of glucose, derived their origin from glycocoll. This confirms the results of Ringer and Lusk<sup>9</sup> which showed that when 20 grams of glycocoll are given to a phlorhizinized dog, 16 grams of extra sugar may be eliminated, indicating the complete conversion of both atoms of carbon in glycocoll into the form of glucose by the organism.

In another dog, to which 20 grams of glycocoll were given, a similar computation shows that 16.33 grams of extra sugar were so eliminated. The details of this experiment need not be given.

Cremer<sup>10</sup> has expressed doubts regarding the origin of so large a quantity of sugar from glycocoll and ascribes the result to the method of calculation. In truth, the method here detailed does not serve when the quantity of extra glucose is to be determined in hourly periods. It is evident from many experiments that ingested glycocoll does reduce the quantity of protein metabolism, and in the above computation it appears that while the average elimination during the fore- and after-periods is 0.724 gram of protein nitrogen hourly, the average of the fourteen hours following glycocoll ingestion amounts to 0.651 of protein nitrogen. It is evidently impossible to measure the intensity of this sparing action in the several hours. If the protein-sparing effect were greatest during the earlier hours of glycocoll ingestion, then the sugar derived from glycocoll would in reality be greater than would appear by any method of calculation that might be used.

Since the sugar of the fore- and after-periods amounted to 2.43 and 2.45 grams per hour, their average of 2.44 grams of sugar

<sup>9</sup> A. I. Ringer and G. Lusk: *Ztschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

<sup>10</sup> M. Cremer: *Med. Klin.*, viii, p. 2050, 1912.

was deducted from the quantity of sugar eliminated hourly after glycoll ingestion in order to determine the "extra sugar" due to the ingestion of this substance. A similar procedure was followed in the case of the nitrogen elimination. Calculated after this fashion, an elimination of 14.47 grams of total extra sugar is obtained. This quantity of sugar is sufficiently close to 16 grams to indicate that the two carbon atoms (and not three out of four contained in two molecules) of glycoll are converted into glucose in the organism.

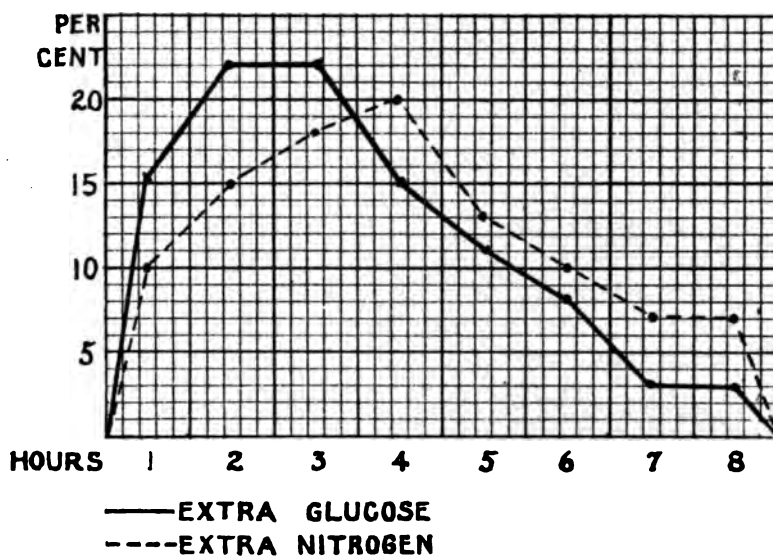


FIG. 1. Curve showing the rate of the elimination of extra sugar and extra nitrogen in per cent after ingestion of 20 gm. of glycoll by a phlorhizinized dog.

According to this calculation, which is based on the hourly elimination of extra glucose after glycoll ingestion, the percentage quantities of glycoll metabolized in eight successive hours amount to 16, 22, 22, 15, 11, 8, 3, and 3 of the total taken. This is more rapid than the corresponding elimination of extra nitrogen, and much more rapid than the increase in nitrogen elimination which occurs after giving 25 grams of glycoll to a normal

dog,<sup>11</sup> under which latter circumstances the maximum increase falls in the fifth hour or later.

The relations are indicated in the accompanying curve (Figure 1), in which the curve of glucose elimination may be taken to indicate the rapidity of the metabolism of 20 grams of ingested glycocoll.

A study of this curve shows that *the calculations of glycocoll metabolism in Professor Lusk's former experiments which were based on the slow elimination of extra nitrogen in normal dogs were erroneous and that the maximum heat production found in the early hours following glycocoll ingestion in reality coincides with the period of maximum glycocoll metabolism.*

Another incomplete experiment which is typical of several in which vomiting took place is the following.

TABLE V.

Dog IV.

*The glucose and nitrogen elimination in hourly periods following the ingestion of 20 grams of glycocoll with 1 gram of Liebig's extract in 200 cc. of water.*

DATE (1914)	PERIOD	GLU- COSE	NITRO- GEN	D.N	EXTRA GLU- COSE	REMARKS
		gm.	gm.		gm.	
	p.m. a.m.					
Mar. 31- Apr. 1	4.20- 9.07	1.46	0.389	3.58		
	a.m.					
Apr. 1	10.00-11.00	*				
	11.00-12.00	6.11	0.700	8.73	4.65	At 10.10 a.m. 20 gm. glycocoll. At 2.15 p.m. vomited.

\* Urine lost.

*In this experiment 28 per cent of the glucose derivable from the glycocoll ingested was eliminated during the second hour. This is significant of the intensity with which the process of the demolition of glycocoll may proceed.*

<sup>11</sup> Lusk: this *Journal*, xiii, p. 166, 1912-13.

*E. Twenty grams of i-alanine.*

Ringer and Lusk<sup>12</sup> gave a phlorhizinized dog 20 grams of *i*-alanine and in two cases found, respectively, 18.76 and 18.78 grams of extra glucose in the urine, indicating a complete conversion of alanine into glucose. Recently, Dakin and Dudley<sup>13</sup> have demonstrated the complete conversion of *l*-alanine into glucose in the phlorhizinized organism.

The following experiment upon a dog weighing 11.7 kgm. was made to determine the rapidity with which sugar is formed after the ingestion of *i*-alanine. The experiment is not perfect for two reasons: (1) the D:N ratio fell from 3.54 in the fore-period to 3.01 in the after-period; and (2) only 70 per cent of the theoretical amount of sugar was eliminated. However, the extra sugar elimination was so large that its calculation is not greatly altered by the change in the D:N ratio. The elimination of 14 grams of extra glucose instead of 20.2 grams called for by theory is to be explained by the inability of the small dog to change chemically the large quantity of alanine administered to it. In previous experiments Lusk<sup>14</sup> has shown that there may be a considerable excretion of unaltered alanine after giving 20 grams to a normal dog weighing 9.7 kgm.

Table VI shows the results obtained after administering 20 grams of *i*-alanine to a phlorhizinized dog.

The extra glucose is computed by the same method as in the glycocholate experiment and amounts to 14.04 gm. If computed by deducting the 3.14 gm. of alanine nitrogen ingested from 5.95 gm. of total nitrogen eliminated during the first eight hours, and multiplying the remainder, or 2.81, by 3.54, which gives 9.95 gm. of glucose derived from protein, and then subtracting this from 24.83 gm. of glucose actually eliminated, the amount of extra glucose derived from the alanine ingested is shown to be 14.88 gm. In this experiment, therefore, only 70 per cent of the theoretical amount of sugar was eliminated.

The relation between the rates of elimination of extra glucose and extra nitrogen after administration of 20 grams of alanine are shown in the curves in Figure 2.

<sup>12</sup> Ringer and Lusk: *loc. cit.*

<sup>13</sup> H. D. Dakin and H. W. Dudley: *this Journal*, xvii, p. 451, 1914.

<sup>14</sup> Lusk: *ibid.*, xiii, p. 169, 1912-13.

It is evident from this curve that the extra glucose elimination following the ingestion of alanine is largely accomplished during the hours immediately after its ingestion.

TABLE VI.

*Dog III. Weight 16 kgm.*

*Elimination of glucose in hourly periods following the ingestion of 20 grams of i-alanine in 210 cc. of water.*

DATE (1914)	PERIOD	GLU- COSE	NITRO- GEN	D:N	EXTRA GLUCOSE		EXTRA NITROGEN		REMARKS
		gm.				per cent		per cent	
Jan. 27-28	p.m. a.m. 5.00- 9.00	1.57	0.450	3.54					
Jan. 28	a.m. 10.00-11.00	2.97	0.642	4.63	1.51	11	0.192	7	At 10.02
	11.00-12.00	4.66	0.901	5.17	3.20	23	0.451	16	a.m. 20
	p.m. 12.00-1.00	4.37	1.006	4.34	2.91	21	0.556	20	gm. i-al-
	1.00-2.00	3.67	0.908	4.04	2.21	16	0.458	16	anine
	2.00-3.00	2.62	0.634	4.13	1.16	8	0.184	7	(3.14 gm.
	3.00-4.00	2.21	0.604	3.65	0.75	5	0.154	6	N).
	4.00-5.00	2.34	0.680	3.45	0.89	6	0.229	8	
	5.00-6.00	1.99	0.573	3.47	0.53	4	0.123	4	
		24.83	5.948						
	6.00-11.00	1.64	0.536	3.05	0.88*	6*	0.431*	16*	
Jan. 28-29	p.m. a.m. 11.00-10.00	1.35	0.45	3.01					
					14.04		2.778		

\* During five hours.

#### *F. Thirteen grams of ethyl lactate.*

Many, as yet unpublished, experiments have been made in this laboratory to determine the influence of the ingestion of ethyl lactate upon the heat production of normal dogs. It seemed desirable to investigate the rapidity of its breakdown in the organism. Theoretical considerations would assume its hydrolysis into alcohol and lactic acid, which latter would be converted into glucose in the phlorhizinized dog and eliminated in the urine as extra sugar.

To determine these relations, 13 grams of ethyl lactate, which on hydrolysis should yield 9.92 grams of lactic acid and 5.07 grams of ethyl alcohol, were given. The results are presented in Table VII.

This observation shows that *the elimination of extra glucose following the ingestion of 13 grams of ethyl lactate is only 41 per*

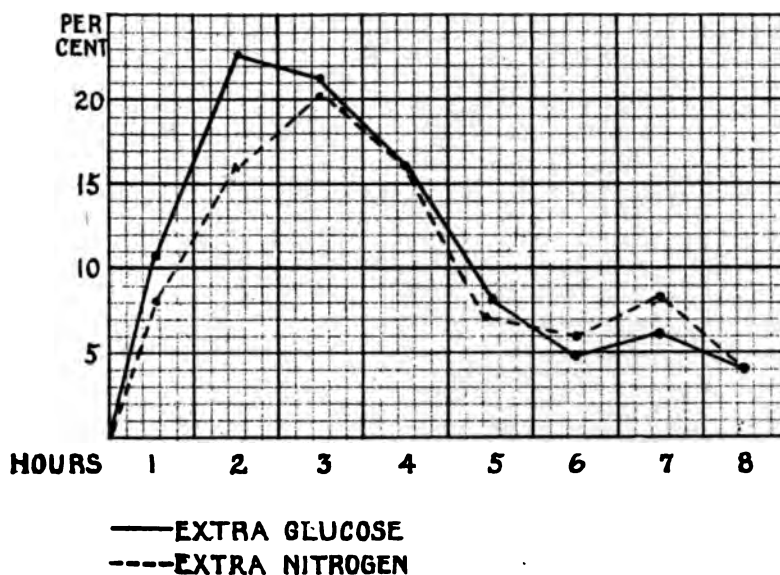


FIG. 2. Curve showing the relative rates of the elimination of extra sugar and extra nitrogen in per cent after the ingestion of 20 gm. of alanine by a phlorhizinized dog.

*cent of the quantity derivable from its lactic acid moiety, and that its elimination is distributed over seven hours. The further fate of ethyl lactate has not been investigated.*

#### IV. DISCUSSION OF RESULTS.

The figures obtained in these experiments make it possible to compare the rates of elimination of extra sugar in successive hours after the ingestion of comparable quantities of glucose, glycocoll, and alanine. Table VIII shows these facts.



TABLE VII.

*Dog III. Weight 9 kgm.**Elimination of glucose in hourly periods following the ingestion of 13 grams of ethyl lactate in 200 cc. of water.*

DATE (1914)	PERIOD	GLU- COSE	NITRO- GEN	D:N	EXTRA GLUCOSE		REMARKS
		gm.	gm.		gm.	per cent	
	<i>p.m. a.m.</i>						
Feb. 16-17	5.00-9.00	0.88	0.265	3.32			
	<i>a.m.</i>						
Feb. 17	10.00-11.00	1.488	0.313	4.75	0.628	16	13 gm. ethyl lactate at 10.10 a.m.
	11.00-12.00	1.760	0.294	5.98	0.900	22	
	<i>p.m.</i>						
	12.00-1.00	1.515	0.262	5.80	0.655	16	
	1.00-2.00	1.360	0.257	5.30	0.500	12	
	2.00-3.00	1.372	0.280	4.89	0.512	12	
	3.00-4.00	1.322	0.294	4.49	0.462	12	
	4.00-5.00	1.274	0.276	4.60	0.414	10	
	5.00-6.00	0.574	0.123	4.66			
					4.071		
	6.00-11.00	0.85	0.275	3.07			

TABLE VIII.

*Percentage elimination of glucose per hour after giving glucose, glycocoll, and alanine.*

SUBSTANCE	AMOUNT GIVEN	GLU- COSE THEORY	GLU- COSE ELIMIN- ATED	HRS.									
				1	2	3	4	5	6	7	8	9+	
	gm.	gm.	gm.										
Glucose.....	16	16.	16.15	14	32	22	13	13	4	2			
Glycocoll.....	20	16.	14.47	16	22*	22	15	11	8	3	3		
Alanine.....	20	20.2	14.04	11	23	21	16	8	5	6	4	6	

\* In another case 28 per cent.

TABLE IX.

*Total percentage elimination of extra sugar to the end of each hour after giving glucose, glycocoll, and alanine.*

SUBSTANCE	HRS.								
	1	2	3	4	5	6	7	8	9+
Glucose.....	14	46	68	81	94	98	100		
Glycocoll.....	16	38	60	75	86	94	97	100	
Alanine.....	11	34	55	71	79	84	90	94	100

Table IX, shows what per cent of the total extra sugar has been eliminated up to the end of a given hour.

A study of these figures shows that *the rapidity of the absorption and elimination of glucose ingested in phlorhizin glycosuria is almost the same as the rapidity of the absorption, deamination, syn-*

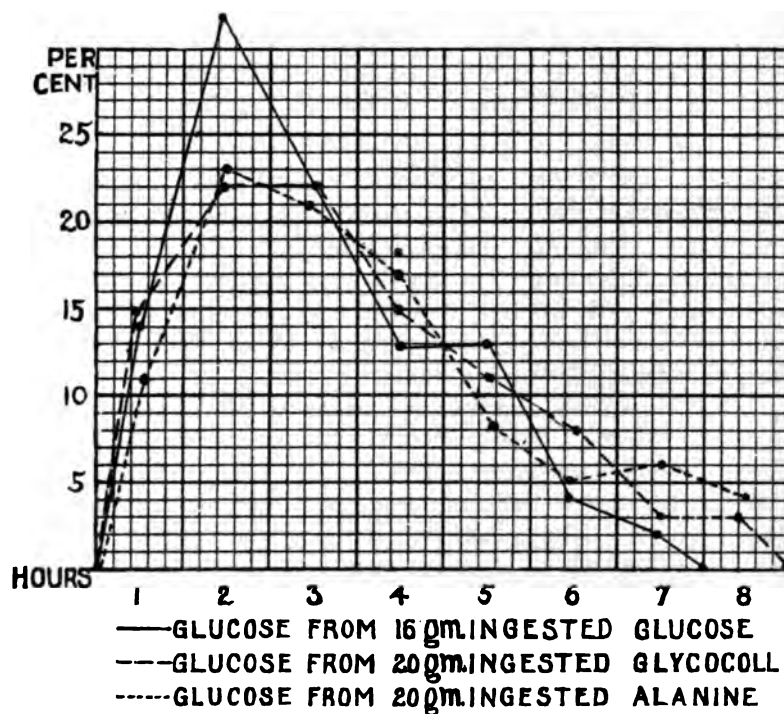


FIG. 3. Curve showing the rate of elimination of extra sugar in per cent after giving glucose, glycocoll, and alanine to phlorhizinized dogs.

*thetic sugar production, and the elimination of such sugar, after ingestion of iso-glucogenic quantities of glycocoll or alanine. Figure 3 illustrates these facts.*

The figures show that a large quantity of glycocoll and alanine is chemically changed during the first hour after their ingestion, whereas Lusk's results, already cited, indicate that there is no increase in the nitrogen output during the first hour following

the ingestion of these substances by a normal dog. Also, in the normal dog, the urinary nitrogen only gradually increases in the immediately following hours, although the phlorhizin experiments show that these are the hours of the maximal chemical change, just as the calorimeter experiments showed them to be the hours of maximal heat production.

These experiments will be employed to interpret new results obtained by the use of the Williams' respiration calorimeter, which are to be published in the Eleventh Paper.

#### SUMMARY.

1. Phlorhization repeated a second or a third time after recovery always leads to a considerable output of sugar during the first hours. The urinary nitrogen does not increase immediately, but the D:N ratio has been found to vary between 5.5 and 17.9, indicating that repeated phlorhization has no effect upon the character of the glycosuria induced.

2. The ingestion of 2 grams of Liebig's extract of beef by the phlorhizinized dog has no perceptible influence on the output of glucose in the phlorhizinized dog.

3. The rapidity of the absorption and elimination of ingested glucose by the phlorhizinized dog is almost the same as the rapidity of the absorption of iso-glucogenic quantities of glycocoll or alanine and of the elimination of the synthetic sugar produced from them after their deamination. The amount of extra sugar elimination in phlorhizin glycosuria appears to be a better index of the intensity of the hourly metabolism of these amino-acids when given to a normal dog than does the nitrogen output under these circumstances.

4. After giving ethyl lactate to a phlorhizinized dog only 40 per cent of the glucose which could have arisen from the lactic acid moiety appeared in the urine.

## ANIMAL CALORIMETRY.

ELEVENTH PAPER.

### AN INVESTIGATION INTO THE CAUSES OF THE SPECIFIC DYNAMIC ACTION OF THE FOODSTUFFS.<sup>1</sup>

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<sup>1</sup> The urinary analyses of 1912-13 were performed by Miss Mary B. Wishart, and those of 1913-14 by Frank A. Csonka.

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### I. INTRODUCTION.

At the International Congress for Hygiene and Demography which took place in Washington, September, 1912, a symposium was held upon the subject of the specific dynamic action of the foodstuffs and was participated in by Rubner, Zuntz, Benedict, and Lusk.

Rubner set forth his well known views upon the subject, and a paper by Zuntz dealt with the problem from his opposed standpoint. These opinions have already been discussed by the writer in the first six papers of this series published in this *Journal* in Volumes XII and XIII and need not here be reiterated. Benedict<sup>2</sup> presented one new especially important experiment in which it was shown that the ingestion of 100 gm. of fructose increased the oxidative processes of a diabetic individual nearly 30 per cent above the basal level without any of the ingested sugar being oxidized, since the respiratory quotient remained at 0.69 as it was before the administration of fructose. Benedict also had reached the conclusion that "diabetics when under conditions of severe acidosis have a much higher metabolism than when the acidosis is mild." Summarizing, Benedict stated: "The amino-acids of protein intermediary metabolism, the glycuronic and similar acids of carbohydrate, and indeed protein intermediary metabolism and the  $\beta$ -oxybutyric acid of the intermediary fat metabolism may each in proportion to the degree of formation stimulate the cells to greater metabolic activity."

At the same meeting in September the writer presented a summary of conclusions based upon work actually accomplished during a period of two years which involved giving individual amino-acids as well as the common foodstuffs to the dog. The work had been long planned, and the papers containing it were in the hands of the editors of this *Journal* before the meeting was held. Lusk's views were thus expressed:

<sup>2</sup> F. G. Benedict: *Transactions of the XVth International Congress on Hygiene and Demography*, ii. p. 394, 1912.

"One may conclude that there are the following forms of metabolism in the quiet or sleeping dog excluded from thermal influences: (1) A basal metabolism in which the cells are nourished by a blood stream which does not receive food from the intestinal tract, but the composition of which is regulated by the organs of the body; (2) a metabolism due to plethora induced by an increased quantity in the blood of carbohydrate or fat metabolites which are being absorbed from the intestine; and (3) a metabolism due to the stimulus of certain incoming amino-acids acting upon the cells. The metabolism of plethora and the metabolism of amino-acid stimulation cannot be added to each other; there is no summation of effect when both influences are brought into action together. In other words, the rhythm of cellular motion induced by the presence of carbohydrate is not further intensified by the stimulus of amino-acids unless the latter alone would accomplish the result." The hope was then expressed that further investigation might bring new evidence in substantiation of these opinions. During the two years which have elapsed since this presentation the writer has devoted much time to the study of these problems along lines which were fully outlined in his mind at the time of the former writing. The results obtained justify a new definition of "amino-acid stimulation."

It will be shown in the present paper that *amino-acid stimulation is in reality due to the stimulus of hydroxy- or keto-acids derived from the deamination of amino-acids and also that a partial summation of effect is brought about by ingestion of glucose and glycocoll*. The rest of the data obtained support and extend the writer's former conclusions.

## II. ERRORS IN FORMER WORK.

### A. Calculations of caloric values of glycocoll and alanine.

Through an unfortunate mistake the heat values for glycocoll and alanine in the Fifth Paper<sup>3</sup> were erroneously given. *The error is not great enough to affect the theoretical deductions.* The corrected values are as follows:

PHYSIOLOGICALLY AVAILABLE CALORIES			
	Per gm. substance	Per gm. N	Per liter O <sub>2</sub>
Glycocoll.....	2.099	11.25	4.685
Alanine.....	3.549	22.04	4.820

The other values given in the table referred to are accurate and have been used in the calculations made throughout this paper.

<sup>3</sup> G. Lusk: this *Journal*, xiii, p. 158, 1912-13.

*B. The discrepancy between direct and indirect calorimetry.*

A considerable difference has been noted between the direct and indirect calorimetry during the first and second hours after giving meat and sugar solutions.<sup>4</sup> The interpretation given to this phenomenon was that there was a considerable warming of the skin of the animal after food ingestion which was not recorded by the rectal thermometer, and hence certain calories which were produced in metabolism were stored in the cutaneous tissue and could not be properly valued. The computation of heat production on the basis of "indirect calorimetry" was therefore always given the preference as the truer index of the intensity of the metabolism. This explanation was erroneous. Only at the end of the present series of experiments was the true cause found. *Materials ingested at a temperature lower than the body temperature absorb heat from the body, the effect of which continues even during the second and third hours.* When glucose is given in 210 cc. of water at a temperature of ordinary tap water, the quantity of "heat calculated" during the second and third hours is three or four calories higher than the quantity of "heat found," whereas *if the sugar solution is given at body temperature there is no discrepancy.* Since the amino-acids were usually given warm with Liebig's extract of beef this technical error is not so apparent as when glucose was given.

*A drink of cold water taken immediately before the calorimeter experiment would upset the agreement between the two methods of computation, so that whenever the two methods do not agree the indirect determination is accepted as the more accurate.* The details will be given in a subsequent discussion (see page 575).

## III. ARGUMENT.

The problems for attack were the following: (1) Since certain individual amino-acids such as glycocoll and alanine stimulate metabolism, will there be a summation of effect if both glycocoll and alanine are given together? (2) Will the rise in metabolism after giving amino-acids be proportional to the quantity of the acid administered? This should follow to accord with Rubner's

<sup>4</sup> H. B. Williams, J. A. Riche, and G. Lusk: *ibid.*, xii, p. 363, 1912.

results after meat ingestion. (3) Can the dynamic effect of amino-acids such as glycocoll and alanine be added to the effect produced by the ingestion of the monosaccharide glucose? (4) What is the specific influence of glucose in contrast to fructose, galactose, and lactose? (5) Is the influence of an oxyacid like lactic acid similar to that of ingested carbohydrate or to that of ingested alanine? In order to test the influence of an oxyacid ethyl lactate was chosen, since it was believed that this would be broken up in metabolism into alcohol and lactic acid. Under such circumstances, what fraction of its influence would be due to alcohol and what to lactic acid? (6) Is the specific dynamic action due to the potential energy of the food itself, or is the effect due to chemical stimulation? This might find its answer in experiments based on the query, What are the relative effects upon heat production in phlorhizin glycosuria of the ingestion of glucose, fructose, glycocoll, and alanine, the carbon content of all of which is completely eliminated as glucose in the urine? Such work should give an answer to the question whether the products of chemical change would stimulate the organism to higher heat production even though these underwent no oxidation whatever.

The present paper deals with the investigation of the problems above described.

#### IV. EXPERIMENTAL PROCEDURE.

The experimental procedure which has become conventional in this laboratory is to give to a dog a standard maintenance diet at five the evening before the experiment and determine the *basal metabolism* the next day, beginning about eighteen hours after food ingestion; having determined this level of heat production the material to be tested is given on another day eighteen hours after food ingestion and the increase in metabolism is noted. The results are calculated according to the method of Zuntz, as in the preceding papers. It should be recalled that the first hour of experimentation represents the second hour after food ingestion.



## V. EXPERIMENTAL WORK.

## A. The summation of glycocoll and alanine.

## a. Basal metabolism of Dog III in 1913.

1913		CALORIES	
		Indirect	Direct
Feb. 26, Exp. 5	1st hr.....	18.73	19.48
	2d hr.....	20.86	20.59
		39.59	40.07
Feb. 27, Exp. 6	1st hr.....	19.71	
	2d hr.....	19.34	
	3d hr.....	20.02	
		59.07	
Mar. 28, Exp. 21	1st hr.....	20.17	19.53
	2d hr.....	19.66	20.60
		39.83	40.13
Average per hr. Exp. 5.....		19.80	20.04
" " " " 6.....		19.69	.....
" " " " 21.....		19.91	20.07
" " " of all experiments.....		19.80	20.05

In all experiments made during this period upon Dog III the basal metabolism was calculated as being 19.8 calories per hour.

b. 5.5 grams of glycocoll and 5.5 grams of alanine. In previous work<sup>5</sup> it was shown that a mixture of 5.5 grams each of glycocoll, alanine, glutamic acid, leucine, and tyrosine, containing together 3.46 grams of nitrogen, when ingested by a dog, caused a rise in metabolism equal to that brought about by the ingestion of 100 grams of beef heart containing 3 grams of nitrogen. It was stated that the various ingredients of the amino-acid mixture each according to its power stimulated the metabolic activity; in other words, that there was a summation of effect.

To test this problem further 5.5 grams of glycocoll or of alanine were given alone and together as shown in the table. For comparison are added the results after giving 50 and 60 grams of glucose. It may be calculated that 11 grams of an equal mixture of glycocoll and alanine yield 10 grams of glucose in metabolism. The results of giving 11 grams of the mixed acids with 50 grams of glucose may therefore be compared with the effect of giving

<sup>5</sup> Lusk: *loc. cit.*, p. 174.

60 grams of glucose. Criticism of this last phase is reserved for another part of the paper (page 586). The following table exhibits the relations discovered.

TABLE I.

*Table showing the influence on the heat production of Dog III of 5.5 grams of glycocoll and 5.5 grams of alanine alone and together, compared with the influence of 50 and 60 grams of glucose and of 11 grams of the mixed amino-acids added to 50 grams of glucose. Results are in three hour periods beginning one hour after food ingestion. Basal metabolism = 19.8 calories per hour.*

DATE (1913)	EXP. NO.	FOOD	CALORIES			CALCULATED INCREASE ABOVE BASAL	
			Indi- rect	Direct	Differ- ence	Calories	Per cent
		gm.					
Mar. 17	13	Glycocoll 5.5	63.80	64.43	+0.63	4.40	7.3
" 7	9	Alanine 5.5	63.99	62.14	-1.85	4.59	7.0
" 10	10	Alanine 5.5	63.51	63.46	-0.05	4.11	7.
" 18	14	Alanine 5.5	62.08	60.72	-1.36	2.68	4.5
		Average.....	63.34	62.69		4.17	7.
" 12	11	Glycocoll 5.5 + alanine 5.5	68.68	67.70	-0.98	9.28	15.5
" 19	15	Glycocoll 5.5 + alanine 5.5	71.63	69.56	-2.07	12.23	20.
		Average.....	70.15	68.63		10.75	18.
" 20	16	Glucose 50	70.35	68.55	-1.80	10.95	18.
" 24	18	Glucose 60	72.36	67.86	-4.50	12.96	21.6
		Average.....	71.35	68.22			
" 21	17	Glucose 50 + glycocoll 5.5 + alanine 5.5	76.15	71.26	-4.89	16.75	28.
" 26	19	Glucose 50 + glycocoll 5.5 + alanine 5.5	77.88	72.71	-5.17	18.48	30.
		Average.....	77.01	71.99		17.61	29.

No data are available to determine the rapidity with which these small quantities of amino-acids are metabolized in the organism. It has been assumed that they are oxidized during the first two or three of the experi-

mental hours, although it is certain from the work of Csonka (see Tenth Paper) that a part of the metabolism has already taken place before the first experimental hour in the calorimeter has been commenced.

*The results obtained show that 5.5 grams of glycocoll raised the heat production 7.3 per cent, 5.5 grams of alanine increased it 7 per cent, whereas the two mixed together raised it 18 per cent. Therefore when two amino-acids are given together there is a summation of effect.*

The 11 grams of mixed amino-acids are convertible into 10 grams of glucose. Former work<sup>6</sup> has shown that ingestion of 20 grams of glucose has little or no effect on metabolism. Since 50 grams of ingested glucose cause the same increase of 18 per cent as do 11 grams of the mixed amino-acids, it appears that the amino-acids themselves, or their intermediary metabolites, are more powerful excitants to increased metabolism than are the metabolites of glucose.

*B. The administration of glycocoll and alanine in different quantities.*

*a. The basal metabolism of Dog III in 1914.* Dog III passed the summer months of 1913 in the laboratory, and during the second winter of experimentation the level of the basal metabolism was found to be considerably lower than that of the winter before. The following summer the dog lived in and around a private stable in the country and returned to the laboratory in excellent physical condition. Table II shows the variations in the basal metabolism during 1914. The "standard diet" had been given in each case eighteen hours before the experiment began.

*The effect of menstruation on the basal metabolism is shown in Experiments 31 and 32 as increasing the production of heat.* On the day previous to menstruation the nitrogen elimination was lower than at any time during the periods of experimentation, and then during the period of menstruation it rose to a higher level than at any other time, relations which have been observed by Murlin.<sup>7</sup>

<sup>6</sup> Lusk: *loc. cit.*, p. 31.

<sup>7</sup> J. R. Murlin: *Am. Jour. Physiol.*, xxvii, p. 193, 1910-11.

TABLE II.  
*Basal metabolism of Dog III in 1914.*

DATE	EXP. NO.	WEIGHT kgm.	NO. OF HRS.	R. Q.	CALORIES			N IN URINE	REMARKS
					Indirect	Direct	Calculated per hr.		
(1913)									
Dec. 13	31	12.1	3	0.81	53.36	53.11	17.79	0.078	One day before menstruation.
" 17	32	12.2	3	0.79	55.55	54.91	18.52	0.182	During menstruation.
(1914)									
Jan. 6	33	12.4	3	0.83	49.13	48.64	16.38	0.150	
" 14	38	12.3	3	0.90	49.39	46.68	16.46	0.124	
" 16	39	12.3	3	0.86	50.60	50.56	16.86	0.131	
Feb. 10	49	12.6	3	0.93	49.88	49.31	16.63	0.116	
" 28	58	12.8	3	0.89	51.53	50.46	17.18	0.069	
Mar. 16	69	13.2	3	0.82	57.48(?)*	53.64	19.16	0.129	After high carbohydrate.
Apr. 1-2	73, 74	12.2	3	0.88	47.88		15.96	0.096	Normal after phlorhizin.
" 28	75	12.8	2	0.82	35.44	32.53	17.72	0.080	
May 4	79	12.8	2	0.79	38.27(?)*	34.50	19.13	0.117	After high carbohydrate.
Oct. 10	84	12.7	3	0.83	60.88	59.96	20.29	0.136	

\* The query is inserted (1) because of the low respiratory quotients and (2) because the direct and indirect calorimetry were at variance.

Five determinations of the basal metabolism between January 6 and February 28 showed that the average heat production per hour was *16.70 calories*. This is 16 per cent lower than the basal metabolism of the year before, although there was little change in the body weight of the animal. The cause of the change in metabolism is not apparent as the diet was the same. It appears probable, however, that prolonged life in the restricted space of a dog cage may reduce the tone of the muscles, and, therefore, the heat production. The average respiratory quotient of these five experiments is 0.88.

The metabolism which was determined on the day immediately after the disappearance of sugar from the urine at the end of a period of phlorhizin glycosuria indicated the lowest heat production obtained at any time, or 15.96 calories per hour. In this case the dog had fasted and received phlorhizin injections daily during five days and then the standard diet was given for thirteen days. The nitrogen of the ingesta and egesta during the experimental period was determined to be:

	N INGESTED gm.
Standard diet 13 days.....	68.2
Glycocoll and alanine.....	5.5
	73.7
	N EGESTED
Urine and feces 18 days.....	92.4
Difference to body.....	-18.7 gm. N.

Rubner<sup>8</sup> estimates that the dog contains 30 grams of N per kgm. of body weight. If the dog which weighed 13.2 kgm. at the beginning of the period was thus constituted it would have contained 396 grams of nitrogen, whereas at the end its nitrogen content would have been 18.6 grams less, or 377.4 grams. In other words, it contained 5 per cent more N at the beginning than at the end. The metabolism of 16.70 calories was 5 per cent higher at the beginning than was that of 15.96 calories at the end, although the dog had the same body weight on both occasions. The following table illustrates the various factors.

<sup>8</sup> M. Rubner: *Ztschr. f. Hyg.*, lxvi, p. 44, 1908.

TABLE III.

*Table showing variations in hourly basal metabolism in Dog III.*

EXP. NO.	DATE	WEIGHT	CALORIES PER HR.		CALORIES PER SQ. METER OF SURFACE (MEER)	INCREASE ABOVE THE MINIMAL	CONDITION
			Indirect	Direct		per cent	
	(1913)	kgm.					
5	Feb. 26	11.5	19.80	20.03	34.7	+29	Pre-cage life.
	(1914)						
39	Jan. 16	12.3	16.86	16.85	28.2	+ 5	Prolonged confinement.
73-74	Apr. 1-2	12.2	15.96	.....	26.9		Loss of flesh.
84	Oct. 10	12.7	20.29	19.99	32.5	+21	After country life.

The dog spent the summer of 1914 in the country and returned to the laboratory in a condition of good muscular strength. The basal metabolism had again risen to its original level. The results in Experiments 5 and 84, which indicate an hourly heat production of 34.7 and 32.5 calories per square meter of surface, were obtained at an interval of twenty months from each other, and correspond with values in Dog I (weight, 13.8 kgm.) and Dog II (weight, 9.3 kgm.) of 31.6 and 32.6, respectively.

It appears certain that *prolonged confinement in a cage will, after a time, greatly reduce the basal metabolism though there be no loss of weight. Recovery from this condition is achieved through exercise. Also, after loss of body protein, though the body weight remains the same, the basal metabolism obtained under the same dietary conditions is reduced to a lower level.*

*b. Twenty and ten grams of glycocoll.* Csonka, in the Tenth Paper of this series, measured the rapidity of the metabolism of 20 grams of glycocoll as determined by the extra sugar eliminated hourly after giving the substance to a phlorhizinized dog. If 22 per cent of ingested glycocoll is metabolized in the second hour, the nitrogen belonging to it may be calculated by multiplying 3.73 grams of nitrogen contained in 20 grams of glycocoll by 22. From this may be calculated the quantities of heat produced, of oxygen used, and of carbon dioxide evolved. The following table presents these data.

Computation for use in Experiments 48, 55, 56, and 57 in which 20 grams of glycocoll were given.

HRS.	TOTAL GLYCOCOLL N	GLYCOCOLL METABOLIZED PER HR.		METABOLIZED N	EQUIVALENTS		
		per cent			Calories	CO <sub>2</sub>	O <sub>2</sub>
				gm.		gm.	gm.
2	3.73	×	22	= 0.82	9.22	3.86	2.81
3	3.73	×	22	= 0.82	9.22	3.86	2.81
4	3.73	×	15	= 0.56	6.30	2.64	1.91
5	3.73	×	11	= 0.41	4.61	1.93	1.40
6	3.73	×	8	= 0.30	3.37	1.41	1.03
7	3.73	×	3	= 0.11	1.41	0.52	0.38
			81		34.13		

Half of these values were used in Experiments 59 and 61, in which 10 grams of glycocoll were given.

The urinary analyses after giving 10 and 20 grams of glycocoll to Dog III revealed the following relations.

EXP. NO.	FOOD		PERIOD		TOTAL N	NH <sub>3</sub> + UREA N		TOTAL N PER HR.
	gm.		hrs.	min.	gm.	gm.	per cent	
48	Glycocoll 20 + glucose 50		7	9	2.91	2.71	93	0.407
55	" 20 + " 50		7	13	3.15	2.98	95	0.435
56	" 20		7	9	3.19	2.89	91	0.446
57	" 20		7	16	3.41	3.05	90	0.468
59	" 10		7	6	1.95	1.80	92	0.275
61	" 10		7	5	1.92	1.76	92	0.271

These figures show that the nitrogen of the ingested glycocoll was completely eliminated as urea plus ammonia nitrogen and not in the form of glycocoll itself. In the determination of the basal metabolism, Experiment 58, the hourly elimination of total nitrogen was 0.089 gram per hour, of urea plus ammonia nitrogen 0.072 gram. Deducting these quantities of nitrogen from those found in the urinary analyses in Experiments 48 and 55, the following relations appear.

Exp. 48.  $2.91 - 0.64 = 2.27$  gm. extra N from glycocoll.  
 $2.71 - 0.50 = 2.21$  " " urea + NH<sub>3</sub> N from glycocoll.  
 Exp. 55.  $3.15 - 0.71 = 2.44$  gm. extra N from glycocoll.  
 $2.98 - 0.52 = 2.46$  " " urea + NH<sub>3</sub> N from glycocoll.

Therefore, *after ingesting 20 grams of glycoll all the nitrogen derived from it is eliminated in the form of urea or ammonia.*

The other experiments, 56, 57, 59, and 61, show the same relations when it is considered that in these cases 2 grams of Liebig's extract, containing 0.16 gram of N, were given with 20 grams of glycoll, and 1 gram of the extract with 10 grams of glycoll. In the Fifth Paper it was demonstrated that the nitrogen of Liebig's extract is eliminated within four hours after its administration.

*The method of calculation of the hourly metabolism after giving glycoll was as follows.* The protein metabolism of each hour was assumed to be that amount which had been determined in a near-by experiment on the basal metabolism. The glycoll metabolism was estimated on the basis of the rapid elimination of extra sugar in phlorhizin glycosuria. It is fully realized that this method is not absolutely accurate since, for example, Csonka found in different experiments that 22 and 28 per cent of the total quantity of extra sugar may be eliminated in the second hour. However, this fact may explain the differences observed in different experiments as regards the intensity of the heat production during the second hour after glycoll ingestion.

In Experiment 56 the heat production rose in the second hour to 26.3 calories per hour, an increase of 9.6 calories, or 57 per cent above the basal level; whereas in 57 the rise was to 23.7 calories, an increase of 7.0 calories, or 42 per cent.

The following table shows the essential results after giving 20 and 10 grams of glycoll.



TABLE IV.  
*Dog III.*

HRS. AFTER FOOD	EXPERIMENT 56 GLYCOCOLL 20 GM. CALORIES					EXPERIMENT 57 GLYCOCOLL 20 GM. CALORIES					EXPERIMENT 59 GLYCOCOLL 10 GM. CALORIES					EXPERIMENT 61 GLYCOCOLL 10 GM. CALORIES				
	In- direct	Direct	Increase indirect over basal	R. Q.	In- direct	Direct	Increase indirect over basal	R. Q.	In- direct	Direct	Increase indirect over basal	R. Q.	In- direct	Direct	Increase indirect over basal	R. Q.	In- direct	Direct	Increase indirect over basal	R. Q.
2	26.3	24.7	9.6	1.00	23.7	22.5	7.0	0.94	21.5	20.3	4.8	0.95	21.3	22.5	4.6	0.98				
3	21.7	21.2	5.0	0.90	23.5	23.0	6.8	0.96	19.5	21.1	2.8	0.94	20.9	20.4	4.2	0.92				
4	20.4	20.7	3.7	0.88	22.7	21.2	6.0	0.88	19.6	18.7	2.9	0.93	19.8	18.8	3.1	0.91				
5	21.5	20.2	4.8	0.81	21.4	21.0	4.7	0.83	19.7	17.5	3.0	0.84	17.5	18.9	0.9	0.93				
6	23.0	19.4	6.3	0.83	21.1	20.4	4.5	0.79	19.2	19.6	2.5	0.87	19.2	18.6	2.5	0.86				
7	21.0	22.3	4.6	0.85	21.3	23.2	4.5	0.82	18.2	18.2	1.5	0.84	17.3	20.0	0.6	0.94				
	134.2	128.5	34.0	0.88	133.7	131.3	33.5	0.87	117.7	115.4	17.5	0.90	116.0	119.2	15.9	0.92				
	Dif. = 4.4%				Dif. = 1.8%				Dif. = 1.9%				Dif. = 2.8%							

A comparison of the percentile increase in metabolism per hour in Experiment 56 after giving 20 grams of glyocoll with the percentile increase in sugar elimination during the similar six hours after giving the same quantity of glyocoll to a phlorhizinized dog (see page 566) reveals the following relations.

HRS.	TOTAL EXTRA CALORIES	EXTRA CALORIES PER HR.	GLYCOCOLL METABOLIZED PER HR. DURING 6 HRS.
		<i>per cent</i>	<i>per cent</i>
2	7.0	21	27
3	6.8	20	27
4	6.0	18	20
5	4.7	14	12
6	4.5	14	10
7	4.5	13	4
	33.5	100	100

Previous experiments (Fifth Paper) have shown that the increase in metabolism extends into the eighth hour after glyocoll ingestion, which is also the final hour of glyocoll metabolism in phlorhizin glycosuria. Critical consideration of these results leads to the conclusion that *although the hours of the greatest heat production are coincident with the hours of the greatest metabolism of glyocoll, yet the two factors are not exactly proportional, for the increased heat production is evidently higher in the later hours than it would be if exactly proportional to the quantity of glyocoll metabolized.* In other words, the high heat production somewhat outlasts the stimulus.

Another argument in favor of the early oxidation of glyocoll is shown in the fact that the respiratory quotients are higher during the second, third, and fourth hours than in the subsequent hours and usually exceed the average of 0.88 found for the ordinary basal metabolism. This indicates that *glyocoll which yields a respiratory quotient of unity is oxidized during the second, third, and fourth hours after its ingestion.*

These experiments, furthermore, show that after giving 20 grams of glyocoll the heat production amounted to 134.2 and 133.7 calories, or respectively 34.0 and 33.5 calories above the basal metabolism, an average increase of 33.75 calories; whereas after giving 10 grams of glyocoll the heat production amounted

to 117.7 and 116.0 calories or, respectively 17.5 and 15.9 calories above the basal metabolism, an average increase of 16.7. In other words, 20 grams of glycocoll containing 42 nutritional calories increased the metabolism by 33.75 calories, or 33.7 per cent above the basal level, while 10 grams containing 21 nutritional calories increased the metabolism by 16.7 calories, or 16.7 per cent.<sup>9</sup> The increase in total metabolism after glycocoll ingestion is therefore proportional to the quantity of the material ingested.

The increase in the heat production amounts to 80 per cent of the calories ingested. However, one must recall that in the phlorhizinized dog 16 per cent of the ingested glycocoll may be absorbed and the sugar formed from it eliminated in the urine within one hour of its administration, an hour during which no heat measurement can be made by the calorimeter. If the intensity of the glycocoll metabolism is measured by the rate of the elimination of extra sugar in the urine in phlorhizin glycosuria, then during the six hours of actual calorimetric observation only 81 per cent of the ingested substance was oxidized. It may be deduced from this that the extra heat production after giving glycocoll may nearly or quite equal the entire energy which can be furnished by the glycocoll itself.

c. *Twenty and thirty grams of i-alanine.* The methods of experimentation and calculation after giving alanine to a dog were the same as those described in the foregoing glycocoll experiments, although the results obtained cannot be said to be as satisfactory.

The urinary analyses showed the following relations.

EXP. NO.	FOOD  gm.	PERIOD		TOTAL N	NH <sub>3</sub> + UREA N		TOTAL N PER HR.
		hrs.	min.	gm.	gm.	per cent	
36	Alanine 20 + glucose 50	7		2.85	2.10	74	0.41
41	Alanine 20	6	8	2.63	1.83	70	0.43
42	Alanine 20 + glucose 50	7	5	3.45	2.49	72	0.46
43	Alanine 20	7	4	3.02	2.26	75	0.43
44	Alanine 30	7	15	4.15	2.96	71	0.57

It is obvious from this that the percentage quantity of urea plus ammonia nitrogen is much lower than the normal, and much lower than was found after giving glycocoll.

<sup>9</sup> On another occasion the ingestion of 5.5 gm. of glycocoll had increased the metabolism 7.3 per cent (see p. 562).

In Experiment 40, when 70 grams of glucose were given in the food the total nitrogen elimination was 0.138 gram and the urea plus ammonia nitrogen was 0.123 gram per hour. Deducting these values from those found in the urinary analyses in Experiments 36, 41, 42, 43, and 44, the following relations are obtained which indicate the quantity of ingested alanine nitrogen eliminated as well as that which was converted into urea.

EXP. NO.	TOTAL N	NH <sub>3</sub> + UREA N	TOTAL ALANINE N	ALANINE NH <sub>3</sub> + UREA N	ALANINE NH <sub>3</sub> + UREA N TO TOTAL ALANINE N  per cent
36	2.85	2.10	1.88	1.24	66
41	2.63	1.83	1.78	1.11	63
42	3.45	2.49	2.47	1.62	66
43	3.02	2.26	2.04	1.40	70
44	4.15	2.96	3.15	2.07	66

These figures are not far from Csonka's computation that 70 per cent of 20 grams of *i*-alanine ingested by a dog of about 10 kgm. weight is transformed into glucose by the phlorhizinized animal. Csonka found that 11 per cent of the 14 grams of extra sugar found was eliminated in the urine of the first hour, and 79 per cent during the second to seventh hours inclusive, in contrast with 81 per cent during the hours when glycocoll was given.

Since 20 grams of alanine contain 3.14 grams of nitrogen, since 70 per cent was metabolized, and since Csonka's figures indicate the rate of metabolism, one may arrive at the following data through use of the table on page 557.

*Computation for use in Experiments 36, 41, 42, 43, in which 20 grams of alanine were given.*

HRS.	TOTAL ALANINE N	ALANINE METABOLIZED PER HOUR		METABOLIZED N	CALORIES	EQUIVALENTS	
		per cent	gm.			CO <sub>2</sub>	O <sub>2</sub>
2	3.73	× 23	× .70	= 0.50	11.02	3.95	3.46
3	3.73	× 21	× .70	= 0.46	10.14	3.63	3.17
4	3.73	× 16	× .70	= 0.28	6.17	2.20	1.92
5	3.73	× 8	× .70	= 0.18	3.97	1.37	1.20
6	3.73	× 5	× .70	= 0.11	2.42	0.88	0.77
7	3.73	× 6	× .70	= 0.13	2.87	1.04	0.91
	79				36.59		

TABLE V.  
Dog III.  
*Influence of the ingestion of 20 and 30 grams of L-alanine upon the heat production.*

HRS. AFTER FOOD	EXPERIMENT 41 ALANINE 20 GM.				EXPERIMENT 43 ALANINE 20 GM.				EXPERIMENT 44 ALANINE 30 GM.			
	CALORIES		R. Q.		CALORIES		R. Q.		CALORIES		R. Q.	
	Indirect	Direct	Increase indirect over basal		Indirect	Direct	Increase indirect over basal		Indirect	Direct	Increase indirect over basal	
2	22.7	24.6	6.0	0.97	21.5	21.3	4.8	0.92	23.8	23.2	7.1	0.90
3	25.2	24.8	8.5	0.92	20.8	21.8	4.1	0.88	25.2	22.9	8.5	0.83
4	21.3	20.6	4.6	0.90	20.4	18.5	3.7	0.87	20.9	20.2	4.2	0.83
5	20.3	19.8	3.6	0.89	19.9	18.9	3.2	0.84	21.7	18.7	5.0	0.79
6	18.0	17.9	1.3	0.93	18.1	17.6	1.4	0.87	19.4	20.5	2.7	0.84
7									18.5	17.6	1.8	0.87
	107.5	107.7	24.0		100.7	98.1	17.2		129.5	123.1	29.3	
	Dif. = 0.2%				Dif. = 2.5%				Dif. = 4.7%			

One and a half times these values were used in calculating the values for use in Experiment 44, when 30 grams of alanine were given. The results after giving 20 and 30 grams of alanine are noted in Table V.

Since the respiratory quotient of alanine is 0.83, little can be deduced from the quotients obtained as to whether alanine was being oxidized or not. It is probable that alanine is constantly deaminized with the production of lactic acid. One can calculate whether the hourly increase in heat production is proportional to the intensity of the metabolism of alanine, after the fashion described for glycocoll, and arrive at the following results.

HRS.	ALANINE 20 GM.			ALANINE METABOLIZED PER HR.	ALANINE 30 GM.	
	Extra calories per hr.				Extra calories per hr.	Alanine metabolized per hr.
	Exp. 41	Exp. 43	Average		Exp. 44	
	per cent	per cent	per cent		per cent	per cent
2	25	28	27	31	24	29
3	35	24	29	29	29	27
4	20	22	21	22	15	20
5	15	18	17	11	17	10
6	5	8	6	7	9	6
7					6	8
	100	100	100	100	100	100

According to this analysis it appears that *the intensity of the extra heat production runs closely parallel with the intensity of the metabolism of alanine as calculated on the basis of extra sugar elimination in phlorhizin glycosuria.*

This raises the question whether lactic acid produced in the deamination of alanine does not constitute the effective stimulus to increased metabolism. Of this more later.

It is unfortunate that the increases in heat production in Experiments 41 and 43 after giving 20 grams of alanine should be so different as are 24.0 and 17.2 calories. The reason is not clear. The urinary analyses show essentially similar conditions. It may be possible that the difference is due to a difference in glycogenic function, in that the first occasion followed a day of glucose ingestion, and perhaps, therefore, the lactic acid formed

was less readily converted into glucose than on the second occasion, the day preceding which only the standard diet had been administered.

Since 20 grams of alanine contain 70.98 nutritional calories, of which, however, only 70 per cent was available, it follows that the ingestion of 49.77 nutritional calories caused an increase in metabolism of 24 and 17 calories, or 49 and 35 per cent of the calories in the ingested material, an average of 42 per cent. Similarly, in 30 grams of alanine there are 73.6 available nutritional calories and the increase in heat production following their ingestion was 29.3 calories, an increment of 40 per cent.

Another method of analysis considers that during the six hours of actual experimentation following the ingestion of 30 grams of alanine, 54.9 calories were available from the breakdown of that material. This would indicate that the *29.3 calories of extra heat produced were 53 per cent of the heat derivable from the quantity of alanine metabolized during the period.*

*d. Comparison between the action of glycocoll and of alanine.* The ingestion of 20 grams of glycocoll convertible into 16 grams of glucose caused a rise of 33.7 calories in the production of heat. The ingestion of 30 grams of alanine, which (in these experiments at least) was convertible into 20.3 grams of glucose caused a rise of 29.3 calories.

Using the method of phlorhizin calculation it may be determined that, during a period when 13 grams of glucose might have arisen from glycocoll in metabolism, 33.7 extra calories were liberated, whereas after giving alanine during a period when 16.7 grams of glucose could have been produced, 29.3 extra calories were liberated. *The quantity of extra heat produced is therefore not proportional to the sugar-forming material available.* The

factor  $\frac{\text{extra calories}}{\text{grams glucose}}$  is in the case of glycocoll  $\frac{33.7}{13} = 2.6$ , and

in the case of alanine  $\frac{29.3}{16.7} = 1.7$ , or a relationship between glyc-

cocoll and alanine of 3 to 2. *It is possible that this indicates that the action on the heat production of three molecules of glycollic acid formed from glycocoll in metabolism and producing one molecule of sugar is the same as that of two molecules of lactic acid formed from alanine in metabolism which also produce one molecule of sugar.*

It is interesting to note that the extra heat production during glycoll metabolism may correspond to the entire energy content of the glycoll oxidized, whereas the extra heat production after giving alanine may amount to only 50 per cent of the energy content of the alanine oxidized. In other words, *the specific dynamic action of glycoll is 100 and that of alanine 50.*

*C. The administration of carbohydrate.*

The subject of the metabolism of glucose has already been reported upon in the Third and Fourth Papers. The present series of experiments deals with the comparative behavior of metabolism after giving various saccharides, and with the influence on metabolism of the addition of various substances such as amino-acids and alcohol to a glucose ingest.

*a. Technique of the experiments.* After giving glucose in solution a very great divergence between the heat production as calculated by the methods of direct and indirect calorimetry

TABLE VI.

*Agreement between direct and indirect calorimetry in control tests and in experiments on Dog III between Dec. 13 and Mar. 19.*

KIND OF EXPERIMENT	NO. OF EXPERIMENTS	CALORIES			
		Indirect	Direct	Difference	Difference in per cent
Alcohol check.....	5 in all	296.23	294.38	-2.15	-0.7
Electric check.....	3 in all	149.13	151.20	+2.07	+1.4
Basal.....	31, 32, 33, 38, 39, 49, 58, 69	416.92	407.31	-9.61	-2.3
Basal, phlorhizin.....	70, 71	74.13	74.99	+0.86	+1.2
Glycoll 20 gm.....	44, 56, 57	397.43	382.84	-14.59	-3.7
Glycoll 10 gm.....	59, 61	233.75	234.64	+0.89	+0.4
Alanine 20 gm.....	41, 43	208.11	205.74	-2.37	-1.1
Glucose 50 and 70 gm.	34, 35, 37, 40, 46	550.89	513.02	-37.87	-6.9
Fructose 50 gm.....	64, 66	236.12	218.01	-18.11	-7.7
Glucose 50 gm. + glycoll 20 gm.....	48, 55	285.63	261.30	-24.33	-8.6
Glucose 50 gm. + alanine 20 gm.....	36, 42	275.07	258.34	-16.73	-6.1



invariably occurred, and it was not until the conclusion of these experiments that the cause of this difficulty was ascertained. A similar disparity occurred after giving meat to a dog (Second Paper). The preceding table is a summary of results which were obtained with Dog III between December 13 and March 19.

The large deficit in "calories found" led to a most exact measurement in the autumn of 1914. Alcohol checks and experiments with the dog followed each other in rapid succession, always with the same differences in indirect and direct calorimetry after carbohydrate ingestion.

1914	EXP. NO.		CALORIES		
			Indirect	Direct	Difference
Oct. 9		Alcohol check.....	56.52	56.09	-0.43
" 10	84	Basal metabolism.....	59.96	60.88	+0.92
" 13	85	Glucose 70 gm.....	121.03	116.53	-4.50
" 14		Alcohol check.....	47.27	47.35	+0.08
" 16		Alcohol check.....	43.97	43.37	-0.60
" 17	86	Glucose 70 gm.....	118.25	113.21	-5.04
" 20		Alcohol check.....	24.31	24.34	+0.03
" 21	87	Glucose 70 gm.....	142.70	136.16	-6.54

It appeared from this analysis that contrary to theoretical expectations, the production of fat from ingested carbohydrate must be an endothermic process. *The error of this conclusion, however, was decisively shown by experiments in which 70 grams of glucose were given in the customary 210 cc. of water when the whole was warmed to the body temperature before its administration.* Table VII indicates this.

This table clearly shows that after giving glucose in warm water direct and indirect calorimetry agree closely in hourly periods, whereas when the same material is given in tap water there is a very constant increase in the heat production in order to compensate for the cooling effect exerted upon the body tissues. This is manifested by an increased oxidation which raises metabolism to a higher level (28 calories) during the third hour than would have been reached had the solution been given at the body temperature. It seems astonishing that an increase in metabolism should occur as long as the third hour after giving a cold glucose

TABLE VII.  
*Influence of the temperature of the ingested glucose solution upon metabolism.*  
*Dog III.*

EXP. NO.	Hrs.	GLUCOSE 70 GM. IN 210 CC. OF COLD TAP WATER				GLUCOSE 70 GM. IN 210 CC. OF WATER AT 38°			
		85		86		87		88	
		Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct
		cal.	cal.	cal.	cal.	cal.	cal.	cal.	cal.
	2	24.09	25.07	26.52	24.84	26.77	23.99	25.34	26.12
	3	28.47	26.22	28.31	25.75	28.68	25.48	25.26	25.83
	4	27.26	25.66	25.37	23.50	25.53	25.07	25.21	24.86
	5	22.90	19.63	19.75	19.00	21.64	20.20		
	6	19.52	19.95	19.90	20.12	21.32	20.68		
	7					20.18	20.74		
Total....		122.24	116.53	119.85	113.21	144.12	136.16	75.81	76.81
		Dif. = 5.71		Dif. = 6.64		Dif. = 7.96		Dif. = 1.00	
								119.57	121.18
								Dif. = 1.61	
								75.65	76.06
								Dif. = 0.43	

solution. If one averages the heat relations during the first three experimental hours one obtains the following results.

GLUCOSE IN COLD TAP WATER		GLUCOSE IN WATER AT 35°	
Indirect	Direct	Indirect	Direct
cal.	cal.	cal.	cal.
80.33	75.19	75.92	76.39

*It appears from this that there is an actual increase in the production of heat to compensate for a loss of body heat to a glucose solution ingested when it is cold.*

Experiments with cold water did not show this phenomenon as markedly, perhaps on account of the more rapid absorption of the fluid. The following results were obtained with Dog III. The lower metabolism recorded after cold water ingestion is without significance, as the basal metabolism was falling on account of confinement in a cage.

HRS.	EXP. 84. BASAL METABOLISM WITHOUT WATER			EXP. 89. BASAL METABOLISM 210 CC. WATER AT 16.5°		
	Indirect	Direct	Difference	Indirect	Direct	Difference
2	20.43	20.92	+0.49	19.02	18.85	-0.17
3	19.09	18.76	-0.33	17.40	17.26	-0.14
4	21.36	20.28	-1.08	19.49	17.87	-1.62
	60.88	59.96	-0.92	55.91	53.98	-1.93

To another dog, the basal metabolism of which had been found to be the same when measured by the direct and indirect methods, 210 cc. of water at a temperature of 18° were given with the following results.

HRS.	BASAL METABOLISM 210 CC. WATER AT 18°		
	Indirect	Direct	Difference
2	22.97	22.51	-0.46
3	23.46	22.12	-1.34
	46.43	44.63	-1.80
Hr. before water ingestion.....	20.89	20.94	+0.05

In this dog the quantity of heat found was increased by cold water, and furthermore the heat actually produced was greater than the heat found. From this process the 4.2 calories of extra heat necessary to raise 210 cc. from 18° to 38° might partly have been derived.

If one calculates the increase of metabolism over the basal level after giving glucose in cold water with the increase after giving it in warm water one arrives at the following results.

	AFTER GLUCOSE	BASAL	DIFFERENCE	INCREASE
	cal.	cal.	cal.	per cent
Cold glucose solution	80.33	60.88	19.45	32
Glucose solution at 38°	75.92	60.88	15.04	25

*Therefore in the consideration of all the experiments in which glucose has been given and in which the direct and indirect calorimetry do not exactly agree, the results must be considered as having mainly comparative value.*

It became perfectly evident why calories were irrecoverably "lost" after the ingestion of large quantities of meat, and why the direct and indirect calorimetry almost invariably agreed after the dog had been in the calorimeter between two and three hours. The reason why the experimental figures after giving amino-acids were closer than after giving glucose solutions was because the amino-acids were usually given after warming with Liebig's extract of beef. It must be further noted that if the dog drank water voluntarily just before the experiment the calories directly measured would have been deficient. A decided feeling of chagrin must be admitted by the writer in acknowledging that during a period of four years he had wrongly assumed that the ingestion of material regardless of its temperature would be equalized in the body during a preliminary period of three-quarters of an hour and would exert no influence upon the results.

If the temperature of the tap water used to dissolve the sugar had been 18°, it would have required 4.2 calories to warm 210 cc. of water to a temperature of 38°. This is not far from the number of "lost calories." It would appear therefore that the cool liquid reduces the temperature of the parts into which it comes in contact and that these cooled parts are only very gradually supplied

with calories from metabolism to an extent sufficient to restore the heat abstracted from them.

Quincke<sup>10</sup> reports that in a boy weighing 23 kgm. who had a gastric fistula, the temperature of the stomach was usually 0.12 of a degree higher than that of the rectum. Introduction of 500 cc. of milk at a temperature of 18.5° into the stomach (which had an original temperature of 37.5° before the experiment) resulted in a gradual increase in the temperature of the stomach content, which, however, did not reach within 0.5 of a degree of the rectum until 75 minutes after the food was ingested. The rise of temperature in the stomach is at first rapid, as is indicated below:

5 min.,	21.2°
10 min.,	26.8°
15 min.,	28.5°
25 min.,	31.5°
50 min.,	36.1°
75 min.,	37.0°

Taken in consideration with the experimental data obtained with the calorimeter, it seems that the cool ingested fluid must withdraw calories from the adjacent organs subjected to its influence, which calories are only gradually restored.

This, then, replaces the former explanation<sup>11</sup> as regards the "lost calories," which attempted to show that the skin became relatively warmer during the ingestion of food. This theory always lacked confirmation in the recovery of the heat so stored during the later hours of experimentation.

New experiments carried on by Riche, using electrical resistance thermometers for the simultaneous recording of skin temperatures and rectal temperatures, have shown that after giving 50 grams of glucose in 150 cc. of water, if a sufficiently long preliminary period is allowed, there is no relative rise in skin temperature over that of rectal temperature. These results contradict those of Williams, Riche, and Lusk. The work of DuBois with the Sage respiration calorimeter indicates that in man also, except in the case of the fluctuations in fever, there is no large storage of heat under the skin. It is therefore a pleasure to agree with

<sup>10</sup> H. Quincke: *Arch. f. exper. Path. u. Pharmacol.*, xxv, p. 377, 1889.

<sup>11</sup> Williams, Riche, and Lusk: *loc. cit.*, p. 364.

Benedict and Slack<sup>12</sup> that the rectal thermometer may be relied upon in calorimetric work upon man or animals, even though their general statement that a rise or fall in rectal temperature is accompanied by a corresponding rise or fall in temperature of all other parts of the body has certain exceptions.

*b. The heat production during the conversion of carbohydrate into fat.* The caloric values when carbohydrate is converted into fat have been discussed in the Second Paper. Bleibtreu's formula there given is as follows:

$$270.05 \text{ gm. glucose} = 100 \text{ gm. fat} + 54.6 \text{ gm. H}_2\text{O} + 115.45 \text{ gm. CO}_2$$

If it be assumed that after giving carbohydrate in excess only protein and carbohydrate are oxidized, it may be calculated that for every gram of carbon dioxide eliminated in excess of the amount necessary to produce a non-protein respiratory quotient of unity, 0.9 gram of fat has been laid down.

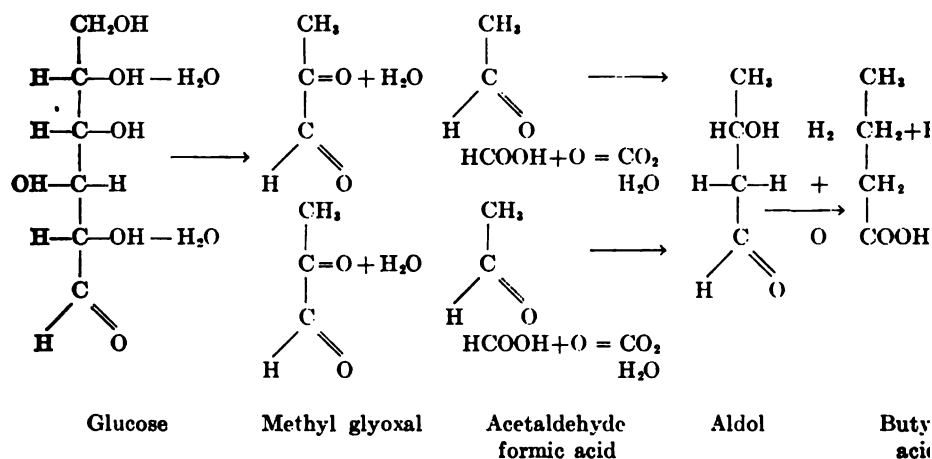
The above reaction is *exothermic*, 4.7 per cent of the energy contained in glucose being eliminated as heat, each liter of carbon dioxide produced in the reaction having a caloric equivalent of 0.803 calories.

Magnus-Levy<sup>13</sup> sets forth the probability of the cleavage of glucose into lactic acid, and of lactic acid into acetaldehyde and the condensation of these aldehyde radicles into fatty acid. In this connection it will be recalled that Neuberg<sup>14</sup> has shown that pyruvic acid  $\text{CH}_3\text{COCOOH}$  is split by yeast enzymes into acetaldehyde with an intense evolution of carbon dioxide gas. Substituting methyl glyoxal for lactic acid or pyruvic acid as the more probable intermediary product, one arrives at the following equation.

<sup>12</sup> F. G. Benedict and E. P. Slack: *Carnegie Institution of Washington, Publication No. 155*, 1911.

<sup>13</sup> A. Magnus-Levy and L. F. Meyer: *Oppenheimers Handb. d. Biochem.*, iv, pt. i, p. 472, 1911.

<sup>14</sup> C. Neuberg and L. Karczag: *Biochem. Ztschr.*, xxxvii, p. 170, 1911.



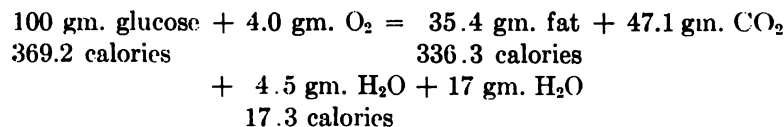
Written in its simplest form this reaction would read:



In the hypothetical production of the higher fatty acids from methyl glyoxal molecules the elimination of  $\text{CO}_2$  from the latter would leave the moiety  $-\text{CH}_2-\text{CH}_2-$ . Whatever the intermediary process is, the reaction for the production of palmitic acid may thus be written:



Calculating that fat is composed of palmitic, stearic, and oleic acids combined with glycerin, Magnus-Levy found that 100 grams of glucose yield, besides water, 35.4 grams of fat, 0.5 gram of hydrogen, and 47.1 grams of  $\text{CO}_2$ . If, however, the hydrogen be oxidized the formula with its caloric values would read as follows:



The reaction is therefore exothermic. In converting glucose into fat with the oxidation of the intermediary hydrogen there is a

calculated loss of 4.2 per cent of heat. These calculated relations are not greatly different from those obtained from the formula first given and used as the basis of calculation. The value of 1 liter of extra  $\text{CO}_2$  would be 0.762 calories in this instance.

The respiratory quotient would be 8.67. After excessive carbohydrate ingestion, therefore, the height of the respiratory quotient will depend upon the extent to which extra  $\text{CO}_2$  molecules are eliminated in the process of fat formation.

The caloric value for the liter of oxygen if used to oxidize hydrogen is 6.17 instead of 5.05 when carbohydrate is oxidized. In Experiment 88 during the third experimental hour, the respiratory quotient was 1.06, and the non-protein quotient 1.12. The "extra  $\text{CO}_2$ " is computed at 0.49 gram and corresponds to a deposition of 0.44 gram of fat ( $0.49 \times 0.9$ ). Using the last quoted formula, 35 cc. of  $\text{O}_2$  might have been used in the intermediary oxidation of hydrogen when 0.44 gram of fat is deposited. In the oxidation of carbohydrate 35 cc. of oxygen would produce 0.17 calories, and in the oxidation of hydrogen, 0.21 calories. It is obvious that such differences are negligible. *The caloric value of the liter of absorbed oxygen remains practically unaffected.*

It appears, therefore, that the special computation of heat production when carbohydrate is converted into fat, which was used in Paper Two, stands the test of further analytical criticism. The method is simply to compute the heat production from the non-protein oxygen absorption as though it involved the oxidation of carbohydrate, and to add thereto the heat produced in the intermediary reactions during the conversion of carbohydrate into fat, as measured by the volume of "extra  $\text{CO}_2$ " eliminated over and above a non-protein respiratory quotient of 1.00. One liter of such "extra  $\text{CO}_2$ " was valued at 0.803 calories as derived from Bleibtreu's formula.

A possible explanation of the high respiratory quotients formed after giving large amounts of carbohydrate is that acids formed in intermediary metabolism might drive carbon dioxide from the blood and from the tissues. This point was studied by Dr. A. L. Meyer<sup>15</sup> who found that the ingestion of 50 grams of glucose by a dog maintained under nutritive conditions which prevailed

<sup>15</sup> A. L. Meyer: Unpublished.



during the experiments with Dog III was without effect on the carbon dioxide content of the blood during the hours immediately following the ingestion of the sugar. Since the carbon dioxide content of the blood is considered a most delicate index to its hydrogen ion concentration, this appears to indicate that *the increase in metabolism after glucose ingestion is not due to acid stimulation.*

That the method of calculation of indirect calorimetry in the presence of respiratory quotients above unity is correct, may be deduced from the experiments in Table VIII. The "uncorrected" heat values represent calculations based on the oxygen absorption alone, while the "corrected" values are those in which the quantity of  $\text{CO}_2$  eliminated in excess of a non-protein respiratory quotient of unity is given a value of 0.803 calories per liter.

*It is evident that after a large ingestion of glucose direct and indirect calorimetry agree closely if the heat value of the carbon dioxide which is evolved in the intermediary transformation of carbohydrate into fat be taken into consideration.*

During the first three hours of Experiments 88, 90, and 91, the calculated heat production was 75.81, 75.30, and 75.64, while the  $\text{CO}_2$  excretion in excess of a non-protein respiratory quotient of 1.00 was 1.07, 0.80, and 1.73 liters. It is therefore apparent that *the intensity of metabolism is not related to the height of the respiratory quotient. The transformation of carbohydrate into fat takes place with the liberation of very little energy, and the height of the total metabolism is scarcely affected by the process.*

*c. Fifty grams of glucose alone and with glycocoll and alanine.* It was shown in the Sixth Paper that when 20 grams of glycocoll were added to a mixed diet consisting of 33 grams of meat plus 50 grams of biscuit meal plus 10 grams of lard, there was no essential change in the height of the heat production above that which glycocoll alone would have induced. Also, that the ingestion of 20 grams of alanine with the same mixed diet caused no increase in metabolism above that induced by the mixed diet alone. It was therefore concluded that the metabolism of plethora induced by a large amount of carbohydrate or fat in the blood stream was not influenced by a summation of effect when amino-acid stimulation was brought into play.

The writer acknowledged at the time that this view needed

TABLE VIII.  
*Dog III.*  
*Metabolism after giving 70 grams of glucose in 210 cc. of water at 38°.*

Hrs.	EXPERIMENT 88				EXPERIMENT 90				EXPERIMENT 91			
	Non-protein R. Q.	Indirect uncor- rected cal.	Indirect corrected cal.	Direct cal.	Non- protein R. Q.	Indirect uncor- rected cal.	Indirect corrected cal.	Direct cal.	Non- protein R. Q.	Indirect uncor- rected cal.	Indirect corrected cal.	Direct cal.
2	1.03	25.24	25.34	26.12	1.05	24.51	24.68	27.46	1.08	24.52	24.78	25.31
3	1.11	24.89	25.26	25.83	1.10	24.54	24.87	23.49	1.14	24.91	25.38	25.63
4	1.12	24.82	25.21	24.86	1.04	25.61	25.75	25.32	1.16	24.98	25.49	25.12
5					1.04	23.56	23.69	23.53				
6					1.05	20.45	20.58	21.38				
		74.95	75.81	76.81		118.67	119.57	121.18		74.41	75.65	76.06

further support, and to this end various experiments were instituted.

*First series of experiments.* Attention is called to the experiments presented in Table I, already given. Here the basal metabolism was 59.4 calories during three hours. The average metabolism after giving 50 and 60 grams of glucose may be compared with that after giving 5.5 grams of glycocoll plus 5.5 grams of alanine, and also with that after giving the same quantities of amino-acids with 50 grams of glucose. The last named would be *isoglucosic* with 60 grams of glucose.

The results may thus be summarized.

TABLE IX.

*Showing the average metabolism in experiments of three hour periods after giving glycocoll, alanine, and glucose separately or together to Dog III. Basal metabolism for three hours = 59.4 calories.*

EXP. NO.	SUBSTANCE GIVEN	CALORIES	
		Indirect	Direct
	gm.		
13	Glycocoll 5.5.....	63.8	64.4
9, 10, 14	Alanine 5.5.....	63.5	62.1
11, 15	Glycocoll 5.5 + alanine 5.5.....	70.2	68.6
16, 18	Glucose 50 and 60.....	71.4	68.2
17, 19	Glucose 50 + glycocoll 5.5 + alanine 5.5.....	77.0	72.0

The disagreement between the direct and indirect computations in Experiments 17 and 19 may be explained as due to the low temperature of the ingested solutions. Under these circumstances the direct measurement of heat production appears to be the more accurate criterion of heat production. Glycocoll and alanine combined caused an increase in the number of calories directly determined of 9.4 calories, or 16 per cent; glucose caused an increase of 8.8 calories, or 15 per cent; whereas glucose plus the two amino-acids caused an increase of 12.6 calories, or 21 per cent. These figures therefore indicate that there is some summation of effect when amino-acids are given with glucose, although the combined influence does not appear as the arithmetical sum of the two influences measured separately.

The question seemed of such importance that a more extended study of it was made at a later date.

*Second series of experiments.* These experiments were instituted on Dog III during 1914 at a time when the basal metabolism was 16.7 calories per hour. All the experiments lasted between five and six hours.

The metabolism of glycocoll and alanine after their ingestion with carbohydrate was assumed to take place at the same rate which occurs when they are ingested alone in phlorhizin glycosuria. The results of the urinary analyses already given on pages 566 and 570 show that the elimination of nitrogen after the administration of glycocoll or of alanine is in no way retarded by the addition of glucose to the diet.

The last two columns of Table X show the constancy of the heat production during the fifth, sixth, and seventh hours after the ingestion of 50 and 70 grams of glucose, of 50 grams of fructose and sucrose, of 50 grams of glucose plus 20 grams of alanine. Only after giving 50 grams of glucose plus 20 grams of glycocoll is there a noticeably higher metabolism during these last hours than takes place after giving glucose alone, and this is to be accounted for through the persistence of the stimulus afforded by glycocoll, elsewhere noticed and commented on (page 569).

The essential answer to the question whether there is a summation of effect upon metabolism when glucose and amino-acids are given together lies in the results obtained during the second, third, and fourth hours after the ingestion of the various materials. It does not matter whether the figures obtained by indirect or by direct calorimetry are used, the conclusion may be decisively drawn.

The following briefly expresses the findings.

MATERIALS INGESTED	INCREASE IN HEAT PRODUCTION	
	Indirect	Direct
	per cent	per cent
Glucose 50.....	30	15
Glucose 70.....	35	23
Glycocoll 20.....	36	33
Alanine 20.....	32	31
Glucose 50 + glycocoll 20.....	56	41
Glucose 50 + alanine 20.....	53	45

TABLE X.  
Showing the increases in metabolism after giving glucose and other carbohydrates, also glucose with amino-acids and amino-acids alone.

EXP. NO.	FOOD GIVEN	HRS. 2, 3, AND 4						HRS. 5, 6, AND 7			
		Indirect		Excess over basal		Direct		Excess over basal		Direct	
		Actual	Average	Actual	Per cent	Actual	Average	Actual	Per cent	Actual	Average
	gm.	cal.	cal.	cal.		cal.	cal.	cal.		cal.	cal.
34	Glucose 50	66.8	64.9	14.8	30	58.2	57.8	7.7	15	54.2**	
46	" 50	63.1				57.4				53.2**	
35	" 70	69.4				62.0				54.6*	54.9
37	" 70	69.8			35	60.6	61.9	11.8	23	56.8*	
40	" 70	64.0				63.0				55.6	
64	Fructose 50	67.9	68.4	18.7	37	62.1	62.4	12.3	24	55.6	
66	" 50	69.8				62.0				58.5*	57.2
77	" 50	67.4				63.2					
67	Galactose 50	61.4	61.4	11.3	22	58.3	58.3	8.2	16	57.6*	
60	Sucrose 50	65.1	67.0	16.9	34	64.0	64.4	14.3	28	54.5*	54.8
68	" 50	69.0				64.7				55.0*	
62	Lactose 50	51.7	51.7	1.6	3	52.0	52.0	1.9	4	49.7	
48	Glucose 50	79.2				70.7				58.2	
	+glycocol 20				56		70.8	20.7	41		59.9
55	Glucose 50	76.7	78.0	27.9		70.9				61.5	
	+glycocol 20									56.7	
36	Glucose 50	75.7	76.1	26.5	53	72.6	72.6	22.5	45	56.4	56.5
42	+alanine 20	76.6				72.7					
	Glucose 50										
	+alanine 20										
56	Glycocol 20	68.2	69.0	18.1	36	66.6	66.7	16.6	33	61.9	63.2
										64.6	
57	Glycocol 20	69.8				66.7				56.4*	
41	Alanine 20	69.2	65.9	15.9	32	70.0	65.3	15.3	31		56.7

In the first place, it is evident that the ingestion of 50 grams of glucose plus 20 grams of glycocoll, which are the glycosic equivalent of 66 grams of glucose, has nearly twice the effect upon metabolism as have 70 grams of glucose. Similarly, after giving 50 grams of glucose plus 20 grams of alanine, which are the isoglucosic equivalent of 70.2 grams of glucose, the effect on the heat production is almost double that following the ingestion of 70 grams of glucose. The stimulus to heat production derived from the ingestion of glycocoll or alanine must therefore be of an entirely different variety from that due to an equivalent amount of glucose.

*After giving 50 grams of glucose plus 20 grams of glycocoll or plus 20 grams of alanine, the increase in metabolism is nearly the sum of the increases brought about by 50 grams of glucose or 20 grams of glycocoll or 20 grams of alanine given separately. These facts are shown in the following table, the results being the same whether the direct or indirect calorimetry method of calculation is used.*

gm.	INCREASE	
	Indirect per cent	Direct per cent
Glucose 50.....	30	15
Glycocoll 20.....	36	33
Sum of both.....	66	48
Glucose 50 + glycocoll 20.....	56	41
Glucose 50.....	30	15
Alanine 20.....	32	31
Sum of both.....	62	46
Glucose 50 + alanine 20.....	53	45

This confirms Rubner's<sup>16</sup> statement that when protein and carbohydrate are given together the effect is always somewhat less than the sum of the effects of the two materials given separately. *The experiments of the author<sup>17</sup> which showed that there was no summation of effect when glycocoll or alanine were added to a mixed diet containing starch, meat, and fat were insufficient to justify the conclusion drawn two years ago that there was no summation of*

<sup>16</sup> Rubner: *Die Gesetze des Energieverbrauchs bei der Ernährung*, Leipsic, 1902, p. 414.

<sup>17</sup> Lusk: *loc. cit.*, p. 185.

effect when glycoll or alanine are given with carbohydrate. This hypothesis was wrong and is withdrawn. The results now obtained are more in harmony with those of Gigon<sup>18</sup> who showed that when protein was given with glucose the carbon dioxide elimination equalled the sum of the amounts which either would have produced alone.

d. *The relative influence of fifty grams of glucose, fructose, galactose, sucrose, and lactose.* Experiments to determine the relative values of different carbohydrates have heretofore been frequently instituted without the simultaneous measurement of the oxygen consumption. Johansson<sup>19</sup> found that in man the increase in the output of carbon dioxide after giving fructose was twice that after giving glucose. Thus, 50 grams of fructose ingested caused an average rise of 13.3 grams of carbon dioxide eliminated, and 50 grams of glucose caused one of only 7.8 grams. To interpret these values as heat equivalents would be erroneous; for when the respiratory quotient is above unity carbon dioxide elimination represents little heat production. Benedict<sup>20</sup> without giving details, reports that the sugars react with increasing force in metabolism in the following order: glucose, sucrose, fructose.

The essential values obtained in the present series of experiments are to be found in Table X. It will be noted that the increase in metabolism takes place during the second, third, and fourth hours, and only in the case of fructose does any considerable increase appear in the fifth and subsequent hours. The relation of the increases in metabolism during the earlier hours after giving 50 grams of carbohydrates to the dog is shown below.

EXP. NO.	SUGAR 50 GM.	AVERAGE R. Q.	HRS. 2, 3, AND 4	
			Increase over basal metabolism	
			Indirect	Direct
			per cent	per cent
34, 36	Glucose.....	1.00	30	15
64, 66, 77	Fructose.....	1.02	37	24
60, 68	Sucrose.....	1.02	34	28
67	Galactose.....	0.93	22	16
62	Lactose.....	0.90	3	4

<sup>18</sup> A. Gigon: *Skand. Arch. f. Physiol.*, xxi, p. 351, 1909.

<sup>19</sup> J. E. Johansson: *ibid.*, xxi, p. 1, 1909.

<sup>20</sup> Benedict: *loc. cit.*

This table confirms the statement of Benedict that glucose, sucrose, and fructose act with increasing effect upon the heat production in the order named. The differences, however, are not very great.

After giving 50 grams of glucose the urine was free from sugar; after 50 grams of fructose the urine reduced copper in Experiment 64, and showed the presence of 2.8 grams of fructose (polarization) in Experiment 68. After the ingestion of 50 grams of cane-sugar, this appeared in the urine to the extent of 0.25 gram in Experiment 60 and in traces in Experiment 67.

*The influence of giving 50 grams of lactose* to the dog was practically negligible. The respiratory quotient remained at 0.90, which is scarcely above the normal average of 0.88 obtained for the basal metabolism. The dog's urine contained 1.2 grams of lactose during the period of experimentation, which shows that the material was absorbed. Evidently lactose was not split into its oxidizable components glucose and galactose. During the fourth, fifth, sixth, and seventh hours the oxygen absorption indicates that a slight increase in metabolism took place. Such an increase might have been induced by the absorption of the products of the bacterial decomposition of lactose. This experiment does not accord with the statement of Weinland<sup>21</sup> that the enzyme lactase is normally present in the intestine of the dog.

*The results after giving 50 grams of galactose* to the dog showed that this sugar is not readily utilized. The increase in metabolism was less than after giving the other two monosaccharides, and the respiratory quotient indicated little increase above the usual basal standard. Also 4.4 grams of galactose appeared in the urine during the period of experimentation. It must therefore be concluded that the organism of the dog is not able to utilize galactose to the extent that it may utilize glucose and fructose. The experiments of Weinland<sup>22</sup> have shown that galactose does not form glycogen as readily as do glucose and fructose.

That after giving glucose, sucrose, and fructose the metabolism as recorded by the respiratory exchange may be increased 30, 34, and 37 per cent above the normal basal metabolism fully explains

<sup>21</sup> E. Weinland: *Ztschr. f. Biol.*, xxxviii, pp. 16 and 607, 1899.

<sup>22</sup> Weinland: *ibid.*, xl, p. 374, 1900.



the results of Grafe<sup>23</sup> who found that the metabolism of the dog was 33 per cent above the fasting level during a day in which carbohydrate to the extent of three to three and a half times the fasting energy requirement was given. The non-protein respiratory quotient under these circumstances rose as high as 1.31, indicating a large conversion of carbohydrate into fat.

There is, however, no evidence in substantiation of Grafe's hypothesis that the continued administration of carbohydrates gradually raises the power of the organism to oxidize carbohydrates. *The power of the organism to oxidize carbohydrate in an individual hour depends largely upon the influx material, and this power may frequently be measured in single hours as over 30 per cent above the level of the basal metabolism.*

*D. The administration of ethyl glycolate, ethyl lactate, and ethyl alcohol.*

The administration of glycollic acid or sodium glycolate invariably resulted in violent vomiting. Lactic acid and lactates also produce the same results. It was suggested to the author that the ethyl esters of glycollic and lactic acid might be a favorable means of liberating glycollic acid and lactic acid within the organism. The use of the substances has necessitated a study of the behavior of ethyl alcohol in metabolism. Although much time has been spent in this work, it has not been satisfactory. In the first place, *ethyl glycolate revealed itself as a poison*. In the second place, during the digestion and metabolism of ethyl lactate, lactic acid is set free to an extent of only one-third the quantity which would theoretically be anticipated.<sup>24</sup> The central idea of these experiments was to determine whether the liberation of lactic acid afforded a stimulus which was akin to that of alanine, from the deamination of which lactic acid arises, or whether its action on metabolism was more feeble, analogous to that of glucose. If the former were the case, then the cause of the increased metabolism after giving alanine might be explained as due to the stimulus of lactic acid.

<sup>23</sup> E. Grafe: *Deutsch. Arch. f. klin. Med.*, cxiii, p. 1, 1913.

<sup>24</sup> Csonka: *this Journal*, xx, p. 539, 1915.

a. *Ethyl glycolate*. On April 2, 1913, Dog II was given 15 gm. of ethyl glycolate in 200 cc. of water at 11.00 a.m. At 12.15 p.m. the dog appeared intoxicated; at 5.00 p.m. ate her food and afterwards vomited much of it. On April 3 the dog showed signs of neuritis, incoordination of the hind legs being especially marked; at 5.00 p.m. she ate three-quarters of her regular diet. On April 4 the condition was greatly improved, though her strength was not quite up to the normal. On April 5 the dog had convulsions, followed by rigidity of the neck muscles, could not drink or retain chloretone given *per os*. On April 7 lockjaw continuous since April 5; 500 cc. of normal saline administered subcutaneously. Death during the night. Autopsy by Dr. Wiggers. Organs congested but otherwise normal. No gross changes, and evidently the symptoms were of nervous origin.

Another dog given the same dose appeared intoxicated shortly afterwards. Five hours after the dose the animal could not stand, but crawled, dragging her paralyzed hind legs. Two days later the dog appeared normal, and ate her food. On the third day she refused food, on the fourth day took some dog biscuit, and thereafter lived without food until the twelfth day. The dog died during the night of this day. Convulsions had evidently taken place, for the tongue was bloody; the gums were swollen, and the muscles of the neck were rigid.

In the temporary recovery of these two cases and the subsequent development of acute symptoms one is reminded of delayed chloroform poisoning. The writer has found no other record of the physiological behavior of ethyl glycolate.

b. *Ethyl alcohol*. Ethyl alcohol has a respiratory quotient of 0.667, much lower than that of carbohydrate or of fat. After giving alcohol the respiratory quotient never sinks to that of alcohol itself, showing that metabolism is at the expense of other materials as well as of alcohol. Rosemann<sup>25</sup> assumes that after giving alcohol the lower respiratory quotient is representative of an oxidation which includes alcohol with carbohydrate and fat, the latter two continuing to be oxidized in the same relative proportion to each other as occurred before taking alcohol. Or it may be assumed that alcohol replaces fat in metabolism and only carbohydrate and alcohol are oxidized. This theory has been adopted in the following work. One liter of inspired oxygen has a heat equivalent of 4.945 calories when used in the oxidation of glucose and of 4.850 when used in the oxidation of alcohol. The following values have been calculated and employed in the work to be described.

<sup>25</sup> R. Rosemann: *Oppenheimers Handb. d. Biochem.*, iv, pt. i, p. 423, 1911.

TABLE XI.

*Showing the heat value of the liter of oxygen when alcohol and glucose are oxidized together.*

R. Q.	CALORIES FOR 1 LITER O <sub>2</sub>		ALCOHOL per cent
	No.	Logarithm	
0.667	4.850		100.
0.715	4.871	0.68762	85.
0.745	4.862	0.68681	76.
0.78	4.873	0.68780	66.
0.82	4.892	0.68949	53.
0.87	4.919	0.69188	39.
0.93	4.928	0.69267	21.
1.00	4.945	0.69417	0

The following two experiments show the effect of giving alcohol to a dog immediately following the determination of the basal metabolism.

TABLE XII.

HRS.	EXPERIMENT 30 Ethyl alcohol 5.8 gm.			EXPERIMENT 51 Ethyl alcohol 9.4 gm.		
	R. Q.	Calories		R. Q.	Calories	
		Indirect	Direct		Indirect	Direct
1	0.84	18.06	17.27	0.88	16.82	14.39
2	0.81	18.05	17.18	0.87	16.82	16.32
Basal metabolism.....	• •	36.11	34.45		33.64	30.71
2	0.78	19.47	18.76	0.76	19.68	19.15
3	0.76	19.80	19.98	0.80	18.82	18.97
4	0.75	19.09	18.99	0.75	*	
Metabolism after alcohol.....		58.36	57.73		38.50	38.12
Increase.....		+8%			+15%	

\* Movement during this hr.

The alcohol was given each time with 200 cc. of water. The administration of 5.8 grams (= 41.1 calories) of ethyl alcohol increases metabolism 8 per cent, and 9.4 grams (= 66.6 calories) increases it 15 per cent. The lowering of the respiratory quotient

indicates the oxidation of alcohol. It appears that *small amounts of alcohol increase the level of the basal metabolism and do not merely replace an isodynamic quantity of fat.* It will be remembered that 50 grams of glucose (= 194.5 calories) increased the metabolism 30 per cent, so that carbohydrate is no mean competitor in this regard. A strict comparison between the two is impossible, because glucose may be rendered inert in the body through transformation into glycogen.

*c. Ethyl alcohol and glucose.* Two experiments were performed after administering 9.4 grams of ethyl alcohol with 50 grams of glucose. The results are shown in the following table.

TABLE XIII.

*Metabolism after the ingestion of ethyl alcohol 9.4 grams and glucose 50 grams.*

HRS.	EXPERIMENT 52			EXPERIMENT 54		
	R. Q.	Calories		R. Q.	Calories	
		Indirect	Direct		Indirect	Direct
2	0.87	21.9	23.2	0.95	23.5	22.4
3	0.87	24.1	20.1	0.91	26.2	23.3
4	0.87	24.3	23.6	0.89	24.3	23.7
5	0.82	21.7	19.4	0.95	17.4	16.2
6	0.84	18.6	17.8	0.83	20.6	20.0
Total.....		110.6	104.1		112.0	105.6
Increase 2, 3, and 4 hrs.....		+40%			+47%	

It will be noticed that the customary discrepancy between direct and indirect calorimetry which occurs after ingestion of a sugar solution is here manifested. In experiments previously mentioned the respiratory quotients during the second, third, and fourth hours after the ingestion of glucose were 1.00 or higher. The respiratory quotients of 0.87 in Experiment 52 indicate that about 40 per cent of the non-protein calories may have been derived from alcohol if the rest were obtained from glucose.

The comparative influence of ethyl alcohol alone and when ingested with glucose may be deduced from the following summary.

	INCREASED HEAT PRODUCTION per cent
Glucose 50 gm. (= 194 calories).....	30
Glucose 70 gm. (= 268 calories).....	35
Ethyl alcohol 9.4 gm. (= 66.6 calories).....	15
Glucose 50 gm. + ethyl alcohol 9.4 gm. (= 261 calories).....	43

It appears from the above table that when 9.4 grams of alcohol are given with 50 grams of glucose, containing in all 261 calories, the increase in metabolism is greater than when the isodynamic quantity of 70 grams of glucose is given. One may also conclude that *the resultant of the ingestion of glucose and ethyl alcohol is nearly the sum of the effect which each produces alone.*

d. *Ethyl lactate alone and with glucose.* These experiments are involved in the doubt as to the fate of ethyl lactate in the organism. Since only 33 per cent of the sugar theoretically derivable from the lactic acid component of the substance is eliminated in the urine of the phlorhizinized dog, one cannot believe that it undergoes complete hydrolysis in the organism. Hence it is not possible to assert whether the influence of the substance on metabolism is wholly due to its cleavage products, alcohol and lactic acid, or whether the substance itself is a potent factor.

The estimation of the heat production after giving ethyl lactate is based upon the fact that when it is oxidized its respiratory quotient would be 0.83 and the value of a liter of oxygen used in its oxidation would be 4.881 calories, or the same value as when with a quotient of 0.87 carbohydrate and fat are oxidized together. No very large error can therefore be incurred by using the customary Zuntz table, which was therefore adopted in these experiments.

The following results were obtained after giving 13 and 15 grams of ethyl lactate and 50 grams of glucose plus 25 grams of ethyl lactate.

TABLE XIV.

DOG III. EXP. 25				DOG IV. EXP. 4			DOG III. EXP. 53		
Ethyl lactate 13 gm.				Ethyl lactate 15 gm.			Ethyl lactate 25 gm. + glucose 50 gm.		
Hrs.	R. Q.	Calories		R. Q.	Calories		R. Q.	Calories	
		In-direct	Direct		In-direct	Direct		In-direct	Direct
2	0.76	21.9	22.9	0.81	24.4		0.99	23.6	24.3
3	0.76	22.0	21.6	0.72	24.1		0.92	24.1	23.0
4	0.79	21.1	19.9	0.77	24.7		0.84	24.1	22.3
5							0.88	22.9	22.6
6							0.95	22.1	22.1
		65.0	64.4		73.2			116.8	114.3
Basal metabolism per hr...		18.0			20.2			16.8	
Increase in 2, 3, and 4 hrs. . . .		+20%			+20%			+42%	

Subjecting this table to analysis in its relation to work before discussed, the following compilation will prove illuminating.

INGESTA		INCREASE IN METABOLISM per cent
Ethyl alcohol 5.8 gm. (= 41.1 cal.)		8
Ethyl lactate 15 gm. (= 83.3 cal.)		20
Ethyl lactate 15 gm. =	{ ethyl alcohol 5.85 gm. = 41.42 cal. lactic acid 11.44 gm. = 41.88 cal.	
		83.30

This shows that the effect of ethyl lactate is far more powerful as a stimulant to metabolism than the effect of its alcohol component alone. *It is probable, though not certain, that liberated lactic acid acts as a stimulant to metabolism.* It has been shown before that 20 grams of glucose scarcely affect metabolism, so that the stimulation to metabolism could not be effected by 11 grams of glucose into which 11 grams of lactic acid might be converted.

When ethyl lactate was given with glucose the heat production rose above that which would have taken place with glucose alone, but no higher than when glucose plus the alcohol moiety of the ethyl lactate was administered. This appears below.

INGESTA	INCREASE IN METABOLISM per cent
Ethyl alcohol 9.4 gm..... (= 66.6 cal.).....	15
Glucose 50 gm. + ethyl alcohol 9.4 gm. (= 261.0 " ).....	43
Glucose 50 gm. + ethyl lactate 25 gm. (= 333.0 " ).....	42
Ethyl lactate 25 gm. = ethyl alcohol 9.75 gm. + lactic acid 19.07 gm.	

It is apparent that in this last case no conclusions of value can be drawn.

#### *E. Metabolism in phlorhizin glycosuria.*

When the writer<sup>26</sup> reviewed the literature of phlorhizin glycosuria in 1912, an experiment of Rubner was cited in which the administration of phlorhizin to a fasting dog caused the metabolism to increase 7 per cent, whereas in two of the writer's own experiments there was no increase in one instance and a 16 per cent increase in another. All these experiments were made during long periods with the Pettenkoffer-Voit respiration apparatus in which the dog was free to move. Rubner's work was published in 1902 and the writer's in 1901 and 1903, at which time the necessity for absolute muscular rest as the basis for comparative measurements was not as fully recognized as now. That only slight differences were then observed was due to the fact that the normal dog is more active and restless than the phlorhizinized dog. Comparative results could only be obtained by determinations made during hours of absolute rest.

The work of Falta, Grote, and Staehelin<sup>27</sup> has shown that the metabolism of the depancreatized dog is increased 41.8 per cent above the normal, and this has been confirmed by Murlin and Kramer<sup>28</sup> who found an increase of 42 per cent above the basal metabolism.

In diabetes mellitus a slightly increased metabolism has been observed. Thus DuBois and Veeder<sup>29</sup> noted an increase in metabolism which approximated 5 per cent. Benedict and Joslin<sup>30</sup>

<sup>26</sup> Lusk: *Ergebn. d. Physiol.*, xiii, p. 372, 1912.

<sup>27</sup> W. Falta, F. Grote, and R. Staehelin: *Beitr. z. chem. Phys. u. Path.*, x, p. 199, 1907.

<sup>28</sup> J. R. Murlin and B. Kramer: *this Journal*, xv, p. 380, 1913.

<sup>29</sup> E. F. DuBois and B. S. Veeder: *Arch. Int. Med.*, v, p. 37, 1910.

<sup>30</sup> F. G. Benedict and E. P. Joslin: *Metabolism in diabetes mellitus*, Washington, 1910; *Metabolism in severe diabetes*, Washington, 1912.

have published two valuable volumes on the subject of diabetes, apparently demonstrating an increase of 15 per cent in metabolism in severe diabetes. The first volume was criticized by Lusk<sup>31</sup> and by Falta<sup>32</sup> both of whom failed to find satisfactory evidence of so large an increase. Lusk computed that the rise did not exceed 5 per cent. The second volume by Benedict and Joslin has not heretofore been critically examined.

The following brief criticism may therefore prove of value. Benedict, Emmes, Roth, and Smith<sup>33</sup> have computed the basal heat production of many normal individuals. Gephart and Du Bois have analyzed this series together with their own normal controls as follows:

NO. OF INDIVIDUALS	WEIGHT IN KGM.	CALORIES PER KGM. PER DAY
10	45-55	28.2
41	55-65	26.2
20	65-75	24.4

These results may be compared with a computation of the heat production of Benedict and Joslin's twenty-two cases of severe diabetes. The figures given by these authors of the caloric values of CO<sub>2</sub> and O<sub>2</sub> were used in the calculations of the heat production for the eight individuals weighing between 45 and 55 kgm. The values are: 1 gram CO<sub>2</sub> = 3.26 calories; 1 gram O<sub>2</sub> = 3.31 calories. A theoretical recalculation showed that the value for CO<sub>2</sub> is correct. Using these values in connection with the eight individuals weighing between 45 and 55 kgm. it was found that the heat production calculated on the CO<sub>2</sub> elimination amounted to 1.23 calories per kgm. per hour, whereas if the oxygen absorbed was used as the basis of computation the heat production was 1.22 calories per kgm. per hour. The CO<sub>2</sub> elimination was therefore used as the basis of valuation in the following table, which shows the heat production in severe diabetes and the variation from the normal, all the determinations in diabetes having been made in the Carnegie Nutrition Laboratory at Boston.

<sup>31</sup> Lusk: *Science*, xxxiii, p. 434, 1911.

<sup>32</sup> W. Falta: *Die Erkrankungen der Blutdrüsen*, Berlin, 1913, p. 441.

<sup>33</sup> F. G. Benedict, L. E. Emmes, P. Roth, and H. M. Smith: this *Journal*, xviii, p. 139, 1914.



*Summary of the heat production of the twenty-two cases of severe diabetes of Benedict and Joslin.*

NO. OF SUBJECTS	AVERAGE WEIGHT	CLASS ACCORDING TO WEIGHT	BASED ON THE CO <sub>2</sub> ELIMINATION			
			Calories per hr.	Calories per kgm. per hr.	Calories per kgm. per day	Increase over normal per cent
4	40.2	35-45	57.74	1.44	34.6	
8	50.9	45-55	52.48	1.23	29.5	4.6
8	58.6	55-65	70.32	1.20	28.8	10.0
2	66.8	65-75	79.85	1.20	28.8	18.0
22						

In one of the two cases in the class weighing 65 to 75 kgm. only one investigation of three nose-piece experiments was made, and therefore the 18 per cent increase in metabolism found in the two patients in this class is to be accepted with caution. For the rest, in eight cases weighing between 45 and 55 kgm., the metabolism is only 4.6 per cent above that of ten normal controls; and in another series of eight cases weighing between 55 and 65 kgm. the average metabolism is 10 per cent above the average metabolism of forty-one normal controls. *From this analysis it appears that in general the heat production in severe diabetes mellitus is increased between 5 and 10 per cent, and not 15 per cent as claimed by Benedict and Joslin, although in certain individual cases a 15 per cent increase may be made out.*

The following computation shows the caloric value to the organism of 1 gram of urinary nitrogen in diabetes when the D: N is 3.65.

Normal value of 1 gm. of urinary N.....	26.51 calories
Deduct glucose $3.65 \times 3.692$ calories.....	13.47 "
Value of 1 gm. of urinary N in diabetes.....	13.04 "

The exchange of respiratory gases concerned in protein metabolism in the diabetic condition would be modified as follows:

	O <sub>2</sub> gm.	CO <sub>2</sub> gm.
Normal respiratory exchange for 1 gm. of urinary nitrogen.....	8.45	9.35
Deduct for 3.65 gm. of unoxidized glucose.....	3.89	5.35
	4.56	4.00

Respiratory quotient = 0.64

These values have been used in the following computations. It is evident that the diabetic respiratory quotient will be between 0.707 for fat and 0.64 for protein. Since fat metabolism predominates, the actual respiratory quotient may be theoretically calculated as 0.69.<sup>34</sup>

The effect of phlorhizin glycosuria upon the metabolism of fasting dogs is shown in Tables XV and XVI.

During the periods of experimentation the dogs were phlorhizinized each day by injecting subcutaneously 1 gram of phlorhizin suspended by grinding in 7 cc. of olive oil after the method of Coolen.

In the three days of fasting and phlorhizin experimentation on Dog II when no sodium carbonate was injected, the respiratory quotients were on each occasion 0.69 and the non-protein respiratory quotients were 0.70. These results were obtained when between 19 and 22 per cent of the total calories obtained were derived from the combustion of those fragments of protein which were not convertible into glucose.

Both tables show a very considerable increase in the heat production after the phlorhization of the individual dogs. The increase did not occur in the hours immediately following the injection of the drug in Dog II. Hence it appears that phlorhizin itself does not cause the rise in metabolism, but rather the withdrawal of oxidizable glucose from the organism. The maximal heat production of this fasting phlorhizinized dog was 27 per cent above the basal metabolism, and 44 per cent above the level of the fasting metabolism.

In Dog III, during the first phlorhization, the maximal increase in metabolism above the basal was 30 per cent, and during the second phlorhization it was 48 per cent, whereas *during the third phlorhization the metabolism rose to 70 per cent above the*

<sup>34</sup> A misconception of the nature of the diabetic respiratory quotient occurs in the writings of Benedict and Joslin (*Metabolism in severe diabetes*, p. 111) as follows: "The quotients here observed with severe diabetes indicate a combustion which is chiefly that of fat. Inasmuch as the relatively small amounts of protein burned *tend to raise the quotient* we find the average values slightly above 0.71 rather than slightly below." On p. 113 the statement appears: "It may be concluded therefore that with the subject lying quietly without food the respiratory quotient in cases of severe diabetes is 0.73."

*basal.* It will be noted that in general the higher the protein metabolism the higher was the total heat production, although no absolute formula can be adduced from these relations.

An interesting point about this vast increase in heat production lies in the fact that it takes place in spite of the fact that there is

TABLE XV.

*Showing the influence of phlorhization upon the metabolism of Dog II in hourly periods.*

DATE (1912)	EXP. NO.	CONDITIONS	URI- NARY D:N	NON- PRO- TEIN R. Q.	CALORIES			INCREASE	
					Protein	In- direct	Direct	Above basal	Above fasting
								per cent	per cent
Apr. 22	51	Basal metabo- lism.....		0.75	3.58	16.00	16.36		
May 6	55	3d fasting day		0.70	3.82	14.93	14.90		
" 7	56	4th fasting day		0.72	3.34	14.04	13.86		
		Same immedi- ately after phlorhizin + Na <sub>2</sub> CO <sub>3</sub> solution sub- cutaneously	7.91	0.77	3.31	14.05	14.18	0	0
" 8	57	5th fasting, 2d phlorhizin day.....	3.68	0.70	3.44	17.71	15.32	11	26
		Same after phlorhizin + Na <sub>2</sub> CO <sub>3</sub> ...	3.61	0.73	3.99	20.26	18.82	27	44
" 10	59	7th fasting, 4th phlorhi- zin day.....	3.40	0.70	4.17	18.83	19.09	15	31
" 11	60*	8th fasting, 5th phlorhi- zin day.....	3.34	0.70	3.68	18.18	17.67	14	30

\* In this experiment phlorhizin was not only given in oil, but also each day 1 gm. was given dissolved in water containing Na<sub>2</sub>CO<sub>3</sub>. Where this latter procedure may have interfered with the respiratory quotient it has been noted in the text.

very little acidosis in phlorhizin glycosuria. Marriott<sup>35</sup> has reported that the maximal concentration of acetone bodies in the blood is found in human diabetes. In pancreas diabetes in the dog, however, there is only the very slightest increase in the ace-

<sup>35</sup> W. M. Marriott: *Jour. Am. Med. Assn.*, lxiii, p. 398, 1914.

tone bodies, as also in phlorhizin glycosuria. In a case of human phlorhizin glycosuria reported by Stanley R. Benedict<sup>36</sup> during a day when the D: N ratio was 3.66 with a meat fat diet, the quantity of urinary acetone bodies amounted to 37 grams. It appears, therefore, that the intensity of the acidosis depends upon

TABLE XVI.

*Showing the influence of phlorhization on the metabolism of Dog III in hourly periods including the influence of 70 grams of ingested glucose.*

DATE	EXP. NO.	CONDITIONS	URI-NARY D: N	NON- PRO- TEIN R. Q.	CALORIES			IN- CREASE OVER BASAL
					Protein	Indi- rect	Direct	
(1913)								<i>per cent</i>
Mar. 28	21	Basal metabolism, 1st phlorhization.....		0.77	3.18	19.91	20.06	
Apr. 25	26	2d day fasting and phlorhizin.....	3.35	0.73	4.19	23.79	21.62	20
" 28	28	5th day fasting and phlorhizin.....	4.05	0.70	5.14	25.96	23.70	30
(1914)								
Feb. 28	58	Basal metabolism... 2d phlorhization...		0.88	2.36	17.14	16.82	
Mar. 18	70	2d day fasting and phlorhizin.....	4.94	0.69	3.90	25.02	25.71	46
" 19	71	3d day.....	4.30	0.72	4.43	25.20	25.04	47
" 20	72	4th day 3d phlorhiz- ination.....	3.82	0.71	5.79	25.24	21.31	48
May 8	80	4th day fasting and phlorhizin.....	3.54	0.70	6.79	29.19		70
" 12	81	8th day fasting and phlorhizin.....	4.22	0.74	7.04	27.10	22.90	60
" 13	82	9th day glucose 70 gm.*.....	22.7	0.73	2.69	25.82	21.07	57

\* Standard diet the night before.

the species of the reacting organism. Since metabolism in human diabetes is increased only 5 or 10 per cent in the presence of severe acidosis, while the metabolism in depancreatized dogs is increased 40 per cent when acidosis is only slightly above the nor-

<sup>36</sup> S. R. Benedict and R. C. Lewis: *Proc. Soc. Exper. Biol. and Med.*, xi, p. 134, 1914.

mal, and by 70 per cent during the very mild acidosis of phlorhizin glycosuria, it is evident that *the increase in metabolism in the diabetic state is in no way proportional to the intensity of the acidosis, and hence the acidosis cannot be the etiological factor of the increased metabolism.*

A much more plausible explanation lies in the three- to five-fold increase above the normal in the quantity of amino-acids metabolized in phlorhizin glycosuria, an explanation suggested by Rubner, although there may be still other contributing factors, such as the increased fat content of the blood.

a. *Glucose ten grams and fructose ten grams.* Johansson<sup>37</sup> gave glucose and fructose to diabetic patients and saw that although glucose when ingested had little effect upon the carbonic acid output, fructose increased it. Since the urinary analyses indicate that portions of both varieties of carbohydrate were oxidized, one must conclude that the results obtained are to be contrasted with similar results in the normal condition.

The experiment of Benedict and Joslin<sup>38</sup> in which 100 grams of fructose were given to a patient with severe diabetes is one to which Benedict<sup>39</sup> has attached fundamental importance. He writes: "After administration of 100 grams of levulose there was a noticeable increase in both carbon dioxide production and oxygen consumption, and, singularly enough, there was not the slightest alteration in the respiratory quotient, showing that the increased metabolism was due wholly to a combustion of materials other than the ingested carbohydrates. The low respiratory quotient of 0.69 was maintained throughout the entire test, although the metabolism at times increased more than 30 per cent of that before taking the sugar. Since throughout the whole period the subject, which was an ideal one, lay absolutely quiet without an extraneous movement of any kind, it can be seen that the increase in metabolism could not be ascribed to external muscular activity."<sup>40</sup>

<sup>37</sup> Johansson: *loc. cit.*

<sup>38</sup> Benedict and Joslin: *Metabolism in severe diabetes*, p. 69.

<sup>39</sup> Benedict: *loc. cit.*, p. 399.

<sup>40</sup> The details may be found in Benedict and Joslin's book on severe diabetes, p. 69.

It is regrettable that this perfectly clear-cut experiment could not be confirmed in phlorhizinized dogs after ingesting 10 grams of levulose.

It was first demonstrated that ingested glucose did not raise metabolism in diabetes. This appears from the following experiment.

TABLE XVII.

DATE (1913)	EXP. NO.	CONDITIONS	URIN- ARY D:N	R. Q.	NON- PRO- TEIN R. Q.	CALORIES		
						Pro- tein	Total indirect	Total direct
Apr. 25	26	2d day fasting and phlorhizin.....	3.35	0.716	0.73	4.19	23.79	21.62
		Same after glucose						
		10 gm. 1st hr.....	9.26	0.75	0.77	3.72	23.93	22.82
		2d hr.....	9.26	0.67	0.68	3.72	20.37	19.51
		Average per hr.....		0.711	0.73		22.15	21.16

The urinary analyses showed the following results:

	TIME	N	D	D:N	EXTRA D
Fasting and phlorhizin ...	10.00 a.m.-1.08 p.m.	1.00	3.37	3.37	
Same after glucose 10 gm.	1.08 p.m.-4.08 p.m.	0.86	7.92	9.26	4.72

It appears from these results that *the ingestion of 10 grams of glucose by a phlorhizinized dog is followed by no increase in metabolism during the second and third hours after its ingestion and during a period in which large amounts of "extra glucose" are eliminated in the urine, i.e., 4.72 grams in three hours.*

*After giving 10 grams of fructose no influence could be observed on the metabolism of the phlorhizinized dog, as appears in the sub-joined table.*

TABLE XVIII.

*The influence of fructose in phlorhizin glycosuria.*

DATE (1914)	EXP. NO.	CONDITIONS	HRS.	URIN- ARY D:N	R. Q.	NON- PRO- TEIN R. Q.	CALORIES	
							Protein	Total indirect
May 8	80	4th day fasting and phlorhizin.....	1	3.54	0.687	0.70	6.79	29.19
			2	3.54	0.733	0.77	6.79	29.58
		Average.....			0.713			29.38
		Same after fructose 10 gm.....	2	6.78	0.695	0.71	5.72	29.57
			3	6.78	0.721	0.74	5.72	29.05
			4	6.78	0.711	0.74	5.72	24.77
		Average.....			0.709			27.80
		8th day fasting and phlorhizin.....	1	4.22	0.719	0.75	7.04	26.57
			2	4.22	0.711	0.74	7.04	27.62
May 12	81	Average.....			0.715			27.10
		Same after fructose 10 gm.....	2	7.31	0.697	0.72	6.08	25.80
			3	7.31	0.697	0.72	6.08	25.80
			4	7.31	0.680	0.70	6.08	24.78
		Average.....			0.692			25.46

The urinary analyses showed the following relations:

EXP. NO.	CONDITIONS	TIME	N	D		D:N	EXTRA D
				Gravi- metric	Polar- ization		
80	Fasting and phlorhizin..	10.15 a.m.- 1.18 p.m.	1.59	5.62	4.84	3.54	
	After fructose 10 gm.....	1.18 p.m.- 5.04 p.m.	1.73	11.76	11.06	6.78	5.64
81	Fasting and phlorhizin..	9.48 a.m.- 12.41 p.m.	1.58	6.67	6.02	4.22	
	After fructose 10 gm.....	12.41 p.m.- 4.51 p.m.	1.95	14.21	13.07	7.31	5.94

It is evident from these data that when 10 grams of fructose are administered to the phlorhizinized dog it is converted into glucose; for comparison between the gravimetric and polariscope

methods shows the same constant difference in result whether fructose was ingested or not. Hence fructose was converted into glucose. In Experiment 81, 60 per cent of the fructose ingested was eliminated in the urine as glucose in four hours and ten minutes. In neither experiment was there any evidence of oxidation of fructose. In Experiment 80, during the fore-period, the respiratory quotient was 0.713, and after fructose ingestion it was 0.709; in Experiment 81 the similar figures were 0.715 and 0.692. Furthermore, there was no increase, but rather a slight decrease in metabolism after the ingestion of fructose.

The results obtained here vary from those obtained by Benedict and Joslin only in the fact that there is little or no change in the heat production in contrast with a rise of 30 per cent obtained by the latter authors. In seeking for the cause one might attribute the discrepancy to the fact that while 10 grams of fructose were given to the dog 100 grams were given to the diabetic man. Ten grams of fructose were given in order to contrast its action with that of 12.5 grams of glycocoll, both of which are convertible into the same quantities of glucose. Since glycocoll had a powerful effect and fructose exercised no effect whatever on metabolism, this course is justified.

The results of Benedict and Joslin may be due to the fact that lactic acid fermentation was set up in the intestines with resultant stimulation of metabolism. Or they may have been brought about by the great hyperglycemia induced. The experiments were not controlled by giving 100 grams of glucose.

Immediately after Experiment 81, the phlorhizinized Dog III was given a portion of the standard diet, and on the following morning 70 grams of glucose. The hour after the ingestion of this material the respiratory quotient (see Experiment 82) was 0.72 instead of over 1.00, as found normally (see page 585), and the heat calculated was 25.82 calories per hour in contrast with 25.46 calories during the hours of fructose ingestion the day before. The D:N was 22.7 and the sugar elimination reached 8.9 grams in two hours and four minutes. This experiment has lately been repeated and during the second and third hours after the ingestion of glucose respiratory quotients of 0.72 were found. The heat production remained unchanged from that determined during two hours previous to the glucose administration. The



hourly sugar elimination rose from 1.5 to 7.9 grams (see Experiment 105, Dog III). Woodyatt<sup>41</sup> has shown that the intravenous injection of glucose in a fasting phlorhizinized animal results in its withdrawal from the circulation and its deposition as glycogen in the muscles which had previously contained only traces of glycogen. One must use this knowledge to explain these experiments. The excess of sugar absorbed is eliminated not only by the kidney, but is also absorbed by the muscles and laid down as glycogen, while the respiratory quotient indicates that glucose could have been oxidized only in minimal quantities. *With all this movement of glucose molecules in the circulation of the phlorhizinized dog, the heat production remains unchanged.*

Since fructose is believed to be broken up into methyl glyoxal molecules which are synthesized into glucose, which in turn may be laid down as glycogen, it is evident that these processes do not stimulate metabolism to higher oxidation. The conclusion must therefore be drawn that the increased metabolism after carbohydrate ingestion is due to the largely increased supply of sugar, or of methyl glyoxal radicles, available for oxidation. When this plethora subsides with the cessation of absorption from the intestines, the glycogenic function regulates the composition of the body fluids and the metabolism falls to the basal level.<sup>42</sup>

b. *Glycocoll, twelve and a half grams.* Before Benedict's work with fructose was published, and before it was known in this laboratory, experiments were instituted in May, 1912, to compare the results of giving 10 grams of glucose to the phlorhizinized dog with those after giving 12.5 grams of glycocoll, which is convertible into 10 grams of glucose. Since administration of glycocoll to fasting phlorhizinized dogs usually produces vomiting, six experiments with four different dogs were instituted before a conclusive result was obtained. It was later found that the addition of 1 gram of Liebig's extract of beef made the solution of glycocoll more palatable without interfering with the results.<sup>43</sup>

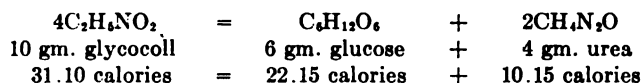
The calculation of the thermal changes which take place when glycocoll is converted into urea and glucose may be estimated in

<sup>41</sup> R. T. Woodyatt: this *Journal*, xiv, p. 441, 1913.

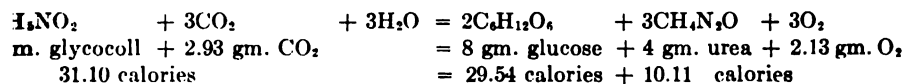
<sup>42</sup> Lusk: *ibid.*, xiii, p. 27, 1912-13.

<sup>43</sup> Lusk: *ibid.*, xiii, p. 159, 1912-13. Csonka: *loc. cit.*

two different ways. The first reaction involves the complete transformation of glycocoll into urea and glucose and accords with the ideas of Cremer.<sup>44</sup>



This corresponds to the endothermic absorption of 1.16 calories. If this reaction took place there would be no change in the respiratory quotient. The work of Ringer and Lusk<sup>45</sup> and the later work of Csonka (Tenth Paper of this series) shows that all the carbon atoms of glycocoll are converted into glucose. This reaction would read:



According to this reaction 2.13 grams of oxygen would be available for the oxidation of fat, and 7.00 calories would be yielded thereby. On one side of the reaction would be 31.1 calories in glycocoll plus 7 calories liberated in the oxidation of fat, or 38.1 calories in all, and on the other side would be 39.65 calories eliminated as glucose and urea in the urine. The net result is an endothermic absorption of 1.55 calories. Both formulae represent essentially the same endothermic relations. The last reaction shows the absorption of the same volume of carbon dioxide for the formation of urea as is the volume of oxygen liberated. The liberation of carbon dioxide resultant upon the oxidation of fat, however, would amount to only 70 per cent of the volume of oxygen employed. The reaction would therefore involve a depression of the respiratory quotient through the reduction in the quantity of carbon dioxide eliminated.

In any event, if the non-protein oxygen be calculated this will give an accurate measure of the quantity of oxygen used in the metabolism of fat irrespective of which of the two formulae is used. It must be assumed that the protein metabolism re-

<sup>44</sup> M. Cremer: *Med. Klin.*, viii, p. 2050, 1912.

<sup>45</sup> A. I. Ringer and G. Lusk: *Ztschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

mains at the same level before and after glycoll ingestion, although this is not strictly true (Csonka, Tenth Paper). Since the results of Csonka show that 20 grams of ingested glycoll are metabolized during the second, third, and fourth hours to an extent of 22, 22, and 15 per cent of the ingesta, deductions concerning the hourly endothermic absorption of heat are based on this hypothesis.

The results of giving 12.5 grams of glycoll to a phlorhizinized dog are presented in the following table.

TABLE XIX.

*Showing the effect of the ingestion of 12.5 grams of glycoll in phlorhizin glycosuria.*

DATE	EXP. NO.	CONDITION	HRS.	URINARY D:N	R. Q.	NON- PRO- TEIN R. Q.	CALORIES		
							Protein	Total indirect	Total direct
(1913)									
Apr. 28	28	Fasting and phlorhizin..	1	3.82	0.700	0.71	5.14	25.72	23.75
			2	3.82	0.685	0.69	5.14	26.21	23.66
			Per hr					25.96	23.70
		Same + glyco- coll 12.5 gm.	3		0.705	0.72	5.14	29.95	25.87
			4		0.709	0.73	5.14	26.01	22.27
			Per hr					27.98	24.07
(1914)									
Mar. 20	72	Fasting and phlorhizin..	2	3.82	0.695	0.71	5.79	25.24	
		Same + glyco- coll 12.5 gm.	2	4.21	0.683	0.69	5.79	31.36	29.19
			3	4.21	0.712	0.73	5.79	26.55	28.62
			4	4.21	0.694	0.71	5.79	29.12	24.67
			Per hr		0.696			29.01	27.49

That glycoll is not oxidized by the phlorhizinized dog is evident by the fact that the usual respiratory quotient of glycoll is 1.00, whereas under the experimental conditions the respiratory

quotient was found to be unchanged. This unchanged respiratory quotient is shown in Experiment 72, it being 0.695 before and 0.696 after glycocoll ingestion, and corresponds with the hypothesis of Cremer that three out of four carbon atoms of glycocoll are converted into glucose. If, however, all the carbon content of glycocoll be convertible into glucose, according to the findings of this laboratory, then the carbon dioxide eliminated in Experiment 72 would have been 330 cc. less during hours 2, 3, and 4 after giving glycocoll than during similar fore-periods without glycocoll. This would reduce the respiratory quotient from 0.696 to 0.680. It is doubtful whether conclusions are to be deduced from experiments involving variations in the respiratory quotients which fall within such narrow limits.

The urinary findings were as follows:

EXP. NO.	CONDITION	TIME	N	D	D:N	EXTRA D
28	Fasting and phlorhizin.....	10.00 a.m.- 1.08 p.m.	1.24	5.01	4.05	
	Same + glycocoll					
	12.5 gm.....	1.08 p.m.- 2.23 p.m.	0.82	4.32	5.29	2.32
		2.23 p.m.- 5.08 p.m.	2.08	9.15	4.37	4.70
						7.02
72	Fasting and phlorhizin.....	9.50 a.m.-12.43 p.m.	1.29	4.92	3.82	
	Same + glycocoll					
	12.5 gm.....	12.43 p.m.- 4.55 p.m.	2.67	11.21	4.21	4.07

It is regrettable that the determinations of heat by the methods of direct and indirect calorimetry do not agree as well as in many other experiments. However, the respiratory quotients accord so narrowly with the theoretical as to leave no doubt that the calculated values are true.

The following recent experiment brings further confirmatory testimony.

*Dog III, March 25, 1915, Experiment 104. Basal phlorhizin metabolism as affected by 20 grams of glycocoll in 210 cc. of water at 38° plus 1 gram of Liebig's extract.*

HRS.		R. Q.	CALORIES	
			Indirect	Direct
1	Basal .....	0.733	23.78	24.53
2	" .....	0.716	23.82	23.84
	Average .....	0.724	23.80	24.18
3	Glycocoll, 20 gm. ....			
4	" .....	0.707	34.21	32.34
5	" .....	0.745	31.65	29.47
6	" .....	0.700	29.24	30.07
7	" .....	0.702	25.99	26.85
	Average .....	0.720	30.27	29.38

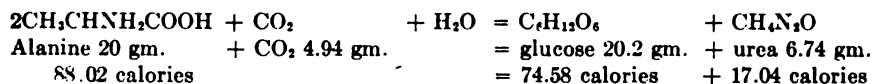
The extra glucose in the urine of five hours was 9 grams.

The experiments show conclusively that *although no glycocoll was oxidized yet there was an increase in heat production after its ingestion in phlorhizin glycosuria*. Since 12.5 grams of glycocoll contain 23.17 physiologically available calories, and since Csonka found that 59 per cent of ingested glycocoll is metabolized during the second, third, and fourth hours after it is given, it may be calculated that 13.67 available calories were contained in the glycocoll converted into sugar during those hours. In Experiment 72 the heat production increased by 11.31 calories above the basal level during a period in which material containing 13.66 available calories was undergoing transformation without oxidation. Here is an increase in metabolism of 83 per cent of the caloric content of the glycocoll. A similar calculation for Experiment 104 shows an increase in metabolism amounting to 91 per cent of the caloric content of the glycocoll transformed during the four hours of experimentation. This contrasts with an extra heat production amounting to nearly 100 per cent of the energy content of glycocoll administered to a normal dog. It is therefore evident that *the cause of the specific dynamic action of glycocoll is independent of the oxidation of glycocoll or of the liberation of its energy content. The increase in metabolism must be due to a stimu-*

*lus to metabolism effected by glycollic acid or other intermediary product.*

That the cause of the specific dynamic action is not due to the amino-acids themselves is evidenced by the following facts. Rubner<sup>46</sup> showed that when ingested protein was retained by the organism it exerted no specific dynamic action. Hoobler has demonstrated that when the protein content of "*Eiweissmilch*" is retained for growth by the infant it causes no increase in metabolism. Miss Wishart (Ninth Paper) finds no increase in the amino-acid content in the muscle of the dog after a large ingestion of meat. Csonka (Tenth Paper) concludes that the increase in heat production coincides with the actual metabolism of glycocoll. It appears therefore that *the amino-acids themselves are not stimuli to metabolism.*

c. *Alanine twenty grams.* The reasons why it is supposed that only 70 per cent of the *i*-alanine used is transformed into glucose and the rest eliminated in the urine have already been given (page 570). The formula for the complete transformation may thus be written.



Lactic acid is here undoubtedly an intermediary product.

The reaction involves an endothermic absorption of 3.60 calories, of which 70 per cent is 2.52. Using Csonka's figures of the rapidity of the metabolism of alanine, one may assume that 16, 20, and 16 per cent of the transformation of alanine into glucose took place during the second, third, and fourth hours after alanine ingestion, and on this basis deductions for the endothermic absorption of heat have been made.

The reaction as given involves the absorption of carbon dioxide for the production of urea. This would reduce the respiratory quotient without, however, affecting the oxygen absorption upon which the calorimetric calculations are based. Assuming Csonka's hourly values for alanine metabolism, one may calculate a withdrawal of carbon dioxide for urea formation, so that the res-

<sup>46</sup> Rubner: *loc. cit.*, p. 256.

piratory quotients in Experiment 70 would be 0.73, 0.72, and 0.70 in the second, third, and fourth hours respectively, instead of 0.68, 0.66, and 0.66 actually found. Since the first two of these revised quotients are too high to be diabetic quotients it may be surmised that a part of the ammonia split off from alanine may be used to neutralize the absorbed acid itself. This may also explain the certain amount of delay in the urea elimination.

The results obtained after the ingestion of 20 grams of *i*-alanine are shown in the following table.

TABLE XX.

DATE (1914)	EXP. NO.	CONDITIONS	HRS.	URINARY D:N	R. Q.	NON- PRO- TEIN R. Q.	CALORIES		
							Protein	Indi- rect	Direct
Mar. 18	70	Fasting and phlorhizin.. Same after al- anine 20 gm.	1	4.94	0.686	0.69	3.90	25.02	25.71
			2	4.71	0.684	0.69	3.90	26.59	28.44
			3	4.71	0.663	0.67	3.90	27.80	24.30
			4	4.71	0.662	0.66	3.90	28.59	25.46
								82.98	78.20
					Per hr.			27.66	26.07

The urinary analyses were as follows:

EXP. NO.	CONDITIONS	TIME	N	D	D:N	EXTRA D
70	Fasting and phlorhi- zin.....	10.00 a.m.-12.55 p.m.	0.88	4.34	4.94	
	Same + alanine 20 gm.....	12.55 p.m.- 4.57 p.m.	2.31	10.90	4.71	4.84

The experiment was performed at the end of the second day of phlorhization, and the high D:N indicates that the body sugar was not fully removed. Alanine itself was not oxidized in the above experiment, for alanine yields a respiratory quotient of 0.83 and would have affected the respiratory quotients had any of it been thus utilized. The low quotients actually obtained have been explained above.

After giving alanine the direct and indirect calorimetry did not

agree. The difference may in part be due to a very great fall in the body temperature of the dog as measured in the rectum. Since the respiratory quotients are theoretically true, the heat calculated from the oxygen absorption has been accepted as correct.

It may be calculated that the total increase in heat production during three hours is 8 calories after the ingestion of 20 grams of alanine in contrast with an increase of 11 calories after 12.5 grams of glycocoll. The latter again appears to be the more powerful stimulant to metabolism. In the case of alanine it is probable that this stimulant is lactic acid which is produced in the process of deamination, and which may readily pass into methyl glyoxal and be converted into glucose (Dakin).

It is apparent from this that both *glycocoll* and *alanine*, even when they are not oxidized and when their energy passes from the phlorhizinized organism in the form of sugar and urea, yield products of metabolism, either oxy- or keto-acids which act as stimuli to induce higher oxidation in that organism. This is the conclusive proof of a true chemical stimulation of protoplasm within the mammalian organism. It explains the specific dynamic action of protein.

#### VI. SUMMARY.

1. After prolonged confinement in a cage without loss of body weight, a dog may manifest a marked reduction in basal metabolism. Recovery from this condition is achieved through exercise.
2. Ingestion of a cold solution of 70 grams of glucose in 210 cc. of water causes a discrepancy between the measurement of heat by the direct and indirect methods which may extend over three or four hours after taking the material. The heat production is increased in order to replace the heat abstracted by the cold solution. To a lesser extent the same phenomenon occurs when cold water is ingested. This explains the disparity between heat found and calculated after meat ingestion noticed in the Second Paper.
3. Glycocoll 5.5 grams increased metabolism 7.3 per cent at a time when alanine 5.5 grams increased it 7 per cent, and when the two mixed together increased it 18 per cent. Therefore, when two amino-acids are given together there is a summation of



effect. Eleven grams of the mixed acids caused the same increase in metabolism as did 50 grams of glucose.

4. Glycocoll 20 grams, containing 42 nutritional calories, increased the metabolism by 33.75 calories, or 33.7 per cent above the basal level; while 10 grams, containing 21 nutritional calories, increased metabolism by 16.7 calories, or 16.7 per cent. The increase is therefore proportional to the quantity ingested. The extra heat production after giving glycocoll is nearly equal to the energy content of the glycocoll administered.

5. After giving 20 and 30 grams of alanine the heat production rose in proportion to the quantity ingested. Extra heat was produced to an extent of 53 per cent of the energy content of the ingested alanine.

6. When the action of glycocoll and alanine is compared, the quantity of extra heat produced is not found to be proportional to the quantity of sugar formed, but there is some evidence to indicate that one molecule of glycollic acid liberated from glycocoll has the same power to increase heat production as one molecule of lactic acid derived from alanine.

7. When carbohydrate is transformed into fat there is a small exothermic elimination of heat, for which due allowance may be made by calculation.

8. After giving glucose 50 grams with glycocoll 20 grams, the increase in the metabolism was almost as great as the sum of the increases induced when each substance was given alone. Alanine 20 grams followed the same law when given with glucose 50 grams. (This nullifies the author's former opinion.)

9. The influence upon heat production of 50 grams of glucose, sucrose, and fructose increases in the order named, which accords with Johansson and with Benedict. The increases in single hours may be over 30 per cent above the basal level.

10. After giving 50 grams of lactose to the dog there was no increase in metabolism nor in the respiratory quotient.

11. After giving 50 grams of galactose there was little increase in metabolism or in the respiratory quotient.

12. Ethyl glycolate is a poison.

13. Ethyl alcohol in small amounts (5.8 and 9.4 grams) increases the level of the basal metabolism and does not merely replace an isodynamic quantity of fat.

14. The resultant of the effect on heat production of ingesting glucose and alcohol together is nearly equal to the sum of the effects which each would have produced alone. The carbohydrate respiratory quotient is greatly reduced.

15. After giving ethyl lactate it is probable, though not certain, that lactic acid acts as a stimulus to higher metabolism.

16. Administration of phlorhizin to a fasting dog may cause an increase in metabolism of as high as 70 per cent above the basal value.

17. Glucose 10 grams or 70 grams and fructose 10 grams have no influence upon the level of heat production in phlorhizin glycosuria.

18. Glycocoll 12.5 grams and alanine 20 grams increase metabolism when they are given to a phlorhizinized dog, though they are not oxidized and their energy content is eliminated in the form of sugar and urea in the urine. Since the maximal effect upon heat production coincides with the period of their maximal metabolism, and since evidence exists to show that amino-acids themselves do not stimulate metabolism (nullifies former opinion) one may conclude that intermediary products such as glycollic acid or lactic acid provide the stimulus. These experiments afford conclusive proof of a true chemical stimulation of protoplasm within the mammalian organism, and offer a logical explanation of the specific dynamic action of protein.

An attempt at a coordinated view of these results is presented in this number of the *Journal* in the form of the presidential address before the Society of Biological Chemists.

100

100

100

100

100

St	BODY	
	Start	
1	37.58	
6		
7		
5	37.02	
4		
9		
3	37.15	
4	37.46	
2		
6		
2	37.53	
2	37.85	
9	37.92	
7	38.12	38

1. The first part of the document is a list of the names of the persons who were present at the meeting. The names are listed in alphabetical order.

2. The second part of the document is a list of the names of the persons who were present at the meeting. The names are listed in alphabetical order.

3. The third part of the document is a list of the names of the persons who were present at the meeting. The names are listed in alphabetical order.

4. The fourth part of the document is a list of the names of the persons who were present at the meeting. The names are listed in alphabetical order.

5. The fifth part of the document is a list of the names of the persons who were present at the meeting. The names are listed in alphabetical order.

		BOD <sub>5</sub>
	Start	
1		
Fe <sub>48</sub>	37.31	
59		
07		
Fe		
M <sub>26</sub>	38.06	
06		
82		
14		
M <sub>10</sub>	37.81	
14		
22		
46		
M <sub>16</sub>	37.93	
87		
67		
70		
M <sub>24</sub>	37.69	
03		
16		
43		
M <sub>31</sub>	37.77	
53		
88		
72		
M <sub>62</sub>	37.92	
50		
44		
56		

11

12

13

BODY TEMPS			
Direct	Start	End	
24.48	38.37	38.2	
23.21		38.1	
20.86		37.8	
68.55			
24.67	38.85	38.4	
23.47		38.2	
23.12		38.0	
20.58		37.9	
91.84			
22.56	38.27	38.1	
22.90		38.0	
22.40		38.0	
20.30		38.1	
88.16			
24.87	38.58	38.1	
25.98		38.1	
21.86		37.1	
21.71		37.1	
94.42			
21.01	38.42	37.1	
20.43		37.1	
19.41		37.1	
60.85			
19.53	37.68	37.1	
20.60		37.1	
40.13			
22.92	38.51	38	
21.57		38	
19.86		38	
64.35			

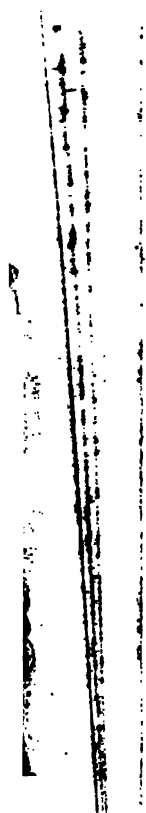




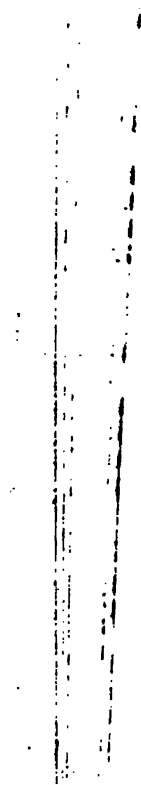
BODY TEMPERATURE			MORNING WEIGHT	BEI
Start	End	Dif.		
			<i>kgm.</i>	
37.82	37.92	+0.10	11.6	Qui
38.19	38.02	-0.17		Qui
	37.90	-0.12		Qui
38.53	38.49	-0.04	10.97	Qui
	38.54	+0.05		Qui
39.41	39.05	-0.36		Dog
	38.63	-0.42		Qui
38.47	38.25	-0.22	11.2	Qui
	38.22	-0.03		Qui
38.53	38.32	-0.21		Occa
	38.29	-0.03		Occa
	38.38	+0.09		Occa
36.74	36.68	-0.06	12.07	Sligh
	36.86	+0.18		Sligh
	36.96	+0.10		Sligh
36.70	36.67	-0.03	12.25	Movl
	36.70	+0.03		Quiet
	36.82	+0.12		Quiet



		BODY TEMPERATURE			MON WE
Indirect	Direct	Start	End	Dif.	
15.16	14.38	37.61	37.58	-0.03	A 12
16.82	17.25		37.77	+0.19	
17.15	17.01		37.91	+0.14	
49.13	48.64				
22.12	18.50	38.27	38.31	+0.04	12
24.17	20.18		38.19	-0.12	
20.53	19.50		38.21	+0.02	
16.72	*17.08		38.19	-0.02	
18.32	*17.60		38.19	±0.00	
101.86	92.86				
22.66	20.67	38.23	38.23	±0.00	12.
23.76	20.82		38.14	-0.09	
22.94	20.45		38.02	-0.12	
19.08	19.12		38.02	±0.00	
17.23	17.26		37.93	-0.09	
105.67	98.32				
24.98	25.49	38.22	38.50	+0.28	12.
25.72	23.52		38.43	-0.07	
24.98	23.60		38.36	-0.07	
22.78	19.66		38.04	-0.32	
20.35	19.49		38.08	+0.04	
20.01	17.55		37.97	-0.11	
138.82	129.31				
22.51	19.58	38.38	38.42	+0.04	12.
23.26	20.91		38.51	+0.09	
24.00	20.16		38.20	-0.29	
20.77	20.01		38.19	-0.01	
18.21	17.86		38.21	+0.02	
108.75	98.52				

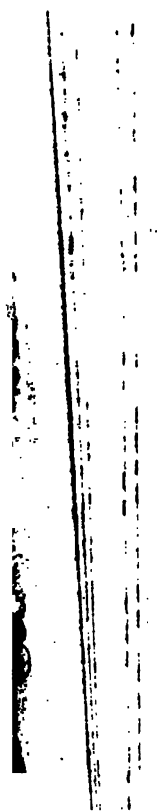


bet	Direct	BODY TEMPERATURE			MORNING WEIGHT
		Start	End	Dif.	
					<i>kgm.</i>
.54	15.78	38.01	38.00	-0.01	12.35
.70	15.26		38.05	+0.05	
.15	15.64		38.09	+0.04	
.39	46.68				
.10	17.73	37.78	37.97	+0.19	12.25
.38	15.96		38.03	+0.06	
.12	16.87		38.04	+0.01	
.60	50.56				
.05	21.45	38.01	38.08	+0.07	12.25
.96	21.27		38.20	+0.12	
.01	20.32		38.09	-0.11	
.45	20.56		38.12	+0.03	
.21	17.63		38.03	-0.09	
.41	17.36		37.96	-0.07	
.09	118.59				
.71	24.63	37.90	38.01	+0.11	12.40
.18	24.80		38.24	+0.13	
.26	20.55		38.14	-0.10	
.34	19.82		38.07	-0.07	
.96	17.86		38.06	-0.01	
.45	107.66				
.52	25.13	38.08	38.37	+0.29	12.40
.64	24.23		38.36	-0.01	
.43	23.30		38.14	-0.22	
.80	19.36		37.77	-0.37	
.00	19.58		37.76	-0.01	
.60	17.43		37.82	+0.06	
.99	129.03				

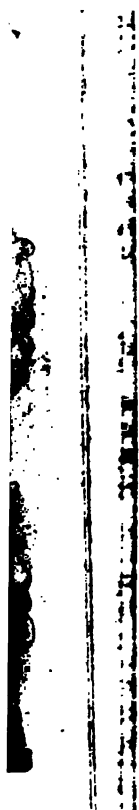


		BODY TEMPERATURE			MO: WI
Indirect	Direct	Start	End	Dif.	
21.47	21.33	38.04	37.91	-0.13	11
20.80	21.77		38.06	+0.15	
20.40	18.53		37.98	-0.08	
19.85	18.89		37.94	-0.04	
18.14	17.56		37.96	+0.02	
20.08	18.64		38.02	+0.06	
120.74	116.72				
23.77	23.22	38.18	38.21	+0.03	11
25.17	22.86		38.15	-0.06	
20.91	20.16		37.86	-0.29	
21.68	18.69		37.70	-0.16	
19.44	20.51		37.87	+0.17	
18.49	17.61		37.80	-0.07	
129.46	123.05				
19.85	18.87	38.20	38.14	-0.06	11
21.15	18.32		38.04	-0.10	
22.08	20.19		38.07	+0.03	
16.19	14.38		37.80	-0.27	
15.95	16.23		37.84	+0.04	
17.30	16.74		37.97	+0.13	
111.52	104.73				
	30.95	37.70	37.82	+0.12	11
	25.47		37.74	-0.08	
	22.28		37.74		
	19.91		37.72	-0.02	
	96.61				



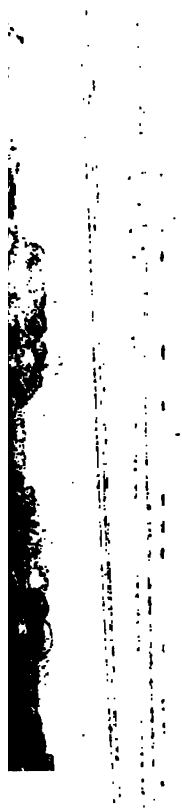


Direct	BODY TEMPERATURE		
	Start	End	Dif.
23.49	38.32	38.88	+0.4
23.82		38.78	-0.1
23.34		38.52	-0.2
19.14		38.22	-0.3
19.14		37.98	-0.2
19.96		37.95	-0.0
128.89			
14.89	38.40	38.09	-0.3
15.72		38.14	+0.0
18.70		38.41	+0.2
49.31			
14.39	37.73	37.58	-0.1
16.32		37.66	+0.0
30.71			
19.15	37.92	37.88	-0.0
18.97		37.90	+0.0
20.38		37.93	+0.0
58.50			
23.17	37.90	38.34	+0.4
20.13		38.02	-0.1
23.63		38.16	+0.2
19.42		37.99	-0.2
17.73		37.77	-0.2
104.08			
24.28	37.86	38.06	+0.2
23.02		38.16	+0.2
22.35		38.12	-0.0
22.58		38.16	+0.0
22.08		38.07	-0.0
114.31			



618

Direct	Sta
22.35	38.
23.32	
23.72	
16.20	
19.97	
20.32	
125.88	
23.78	38.1
23.81	
23.28	
22.29	
20.09	
19.16	
132.41	
24.74	37.1
21.20	
20.73	
20.24	
19.41	
22.23	
128.55	
22.52	37.1
22.96	
21.17	
20.96	
20.42	
23.21	
131.24	
17.95	37.6
15.90	
16.61	
50.46	



No	BODY 73	
	rect	Start
11		
M10.34	37.86	3
1.13		3
8.66		3
7.48		3
9.62		3
8.18		3
5.41		
M12.97	37.61	3
2.57		3
3.47		3
3.89		3
3.41		3
0.31		
M12.50	37.98	3
0.39		3
3.81		3
3.88		3
3.62		3
0.03		3
3.23		
M17.07	37.85	3
3.70		3
3.21	38.27	3
5.51		3
3.12		3
3.11		3
1.72		



DATE	RE NO
1914 Mar. 10	6
Mar. 12	1
Mar. 13	
Mar. 14	
Mar. 16	
JOURNAL	



*Dog III, March 25, 1915, Experiment 104. Basal phlorhizin metabolism as affected by 20 grams of glycocoll in 210 cc. of water at 38° plus 1 gram of Liebig's extract.*

HRS.		R. Q.	CALORIES	
			Indirect	Direct
1	Basal .....	0.733	23.78	24.53
2	" .....	0.716	23.82	23.84
	Average .....	0.724	23.80	24.18
3	Glycocoll, 20 gm. ....			
4	" .....	0.707	34.21	32.34
5	" .....	0.745	31.65	29.47
6	" .....	0.700	29.24	30.07
7	" .....	0.702	25.99	26.85
	Average .....	0.720	30.27	29.38

The extra glucose in the urine of five hours was 9 grams.

The experiments show conclusively that *although no glycocoll was oxidized yet there was an increase in heat production after its ingestion in phlorhizin glycosuria*. Since 12.5 grams of glycocoll contain 23.17 physiologically available calories, and since Csonka found that 59 per cent of ingested glycocoll is metabolized during the second, third, and fourth hours after it is given, it may be calculated that 13.67 available calories were contained in the glycocoll converted into sugar during those hours. In Experiment 72 the heat production increased by 11.31 calories above the basal level during a period in which material containing 13.66 available calories was undergoing transformation without oxidation. Here is an increase in metabolism of 83 per cent of the caloric content of the glycocoll. A similar calculation for Experiment 104 shows an increase in metabolism amounting to 91 per cent of the caloric content of the glycocoll transformed during the four hours of experimentation. This contrasts with an extra heat production amounting to nearly 100 per cent of the energy content of glycocoll administered to a normal dog. It is therefore evident that *the cause of the specific dynamic action of glycocoll is independent of the oxidation of glycocoll or of the liberation of its energy content. The increase in metabolism must be due to a stimu-*

Y TEMPERATURE		MORNING WEIGHT	BEHAVIOR OF DOG	
End	Dif.			
38.41	+0.13	kgm. 12.55	Quiet	Basal phlo days of f.
38.77	-0.11		Quiet	Alanine 20
38.49	-0.28		Quiet	extract +
38.34	-0.15		Moving 1 min.	a.m.
38.40	+0.22	12.25	Quiet	Basal phlo days of f.
38.15	-0.03	12.04	Quiet	Basal phlo days of f.
38.15	±0.00		Quiet	
38.58	+0.13		Quiet	Glycocoll 12
38.58	±0.00		Quiet	extract in
38.19	-0.29		Quiet	
37.26	+0.01	12.14	Quiet	Basal metab appearanc
37.53	-0.14	12.17	Quiet	Basal metab
37.59	+0.06		Quiet	
37.17	-0.20	12.77	Quiet	Basal metab
37.08	-0.09		Quiet	
37.15	-0.34	12.85	Quiet	At 10.00 a.m.
37.69	+0.54		Quiet	cc. water.
37.49	-0.20		Quiet	
37.50	+0.01		Quiet	
37.42	-0.08		Quiet	



BODY TEMPERATURE			MORNING WEIGHT	BEHAVIOR OF DOG
Start	End	Dif.		
37.56	37.36	-0.20	12.84 <i>kgm.</i>	Quiet Moving 1 min.
	37.55	+0.19		
			11.60	Quiet
				Quiet
				Quiet Moved at end Quiet
38.66	38.86	+0.20	9.48	Quiet Moving 1 min.
	38.77	-0.09		
39.02	39.12	+0.10		Quiet
	39.02	-0.10		Quiet
	39.13	+0.11		Quiet
39.09	39.01	-0.08	10.92	Quiet
37.73	37.57	-0.16	12.75	Quiet
	37.51	-0.06		Quiet
	37.56	+0.05		Quiet
37.86	37.94	+0.08	12.70	Quiet
	38.07	+0.13		Quiet
	38.15	+0.08		Quiet
	37.74	-0.11		Quiet
	37.69	-0.05		Quiet



BODY TEMPERATURE			MORNING WEIGHT	BEHAVIOR OF DOG
Start	End	Dif.		
38.08	38.12	+0.04	13.10	Quiet
	38.01	-0.11		Quiet
	37.78	-0.23		Quiet
	37.74	-0.04		Quiet
38.08	38.09	+0.01	12.90	Quiet
	38.10	+0.01		Quiet
	38.14	+0.04		Quiet
	37.84	-0.30		Quiet
	37.80	-0.04	14.05	Quiet
	37.89	+0.09		Quiet
	38.74	-0.06		Quiet
	9.89	+0.15		Quiet
	81	-0.08	14.30	Quiet
	18.85	+0.05		Quiet
	17.26	+0.10		Quiet
	17.87			Quiet
Dep	53.98		14.07	Quiet
	27.46			Quiet
	23.49			Quiet
	25.32			Quiet
	23.53		121.18	Quiet
	21.38			Quiet
	19.57			Quiet
				Quiet

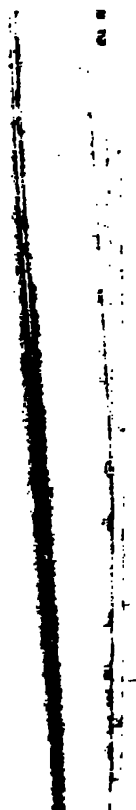
\*Calcul  
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BODY TEMPERATURE			MORNING WEIGHT	BEHA
Start	End	Dif.		
38.75	38.79	+0.04	kgm. 14.27	Quiet
	38.95	+0.16		Quiet
	38.98	+0.03		Quiet
38.79	38.81	+0.02	13.45	Quiet
	38.79	-0.02		Quiet
39.68	39.73	+0.05		Quiet
	39.52	-0.21		Quiet
	39.40	-0.12		Quiet
	39.18	-0.22		Quiet
38.82	39.32	+0.50	13.55	Quiet
				Quiet
				Quiet

TEMPERATURE		MORNING WEIGHT	BEHAVIOR OF DOG
End	Dif.		
38.27	-0.30	kgm 12.7	Quiet
38.55	+0.28		Quiet
38.07	-0.48		Quiet
		12.65	Quiet
			Quiet
			Quiet





## ON THE COLORIMETRIC ESTIMATION OF URIC ACID IN URINE.

By STANLEY R. BENEDICT AND ETHEL H. HITCHCOCK.

(From the Department of Chemistry, Cornell University Medical College,  
New York City.)

(Received for publication, February 8, 1915.)

About three years ago Folin and Macallum<sup>1</sup> described a new micro method for the determination of uric acid in urine, which depends upon a color reaction between uric acid and a specially prepared solution of phosphotungstic acid. Subsequently, Folin and Denis<sup>2</sup> modified the procedure somewhat, introducing a preliminary precipitation of the uric acid by Salkowski's method. These latter investigators also proposed a permanent standard solution for use in making the color comparison for uric acid determinations.

The so called micro methods of analysis are particularly applicable to substances which occur in small quantities, because in such cases a relatively high percentage error, which is apt to occur in micro methods, is of little moment; and, furthermore, because our recognized methods for the determination of such substances are themselves usually subject to considerable error. It would seem, therefore, that a rapid micro method for uric acid determination should be of special service, since the older method for this determination is laborious and not above suspicion regarding its accuracy. Yet the colorimetric procedures suggested for uric acid estimation by Folin and his collaborators do not seem to have met with as general adoption as they appear to merit. Perhaps this is due partially to certain inaccuracies of presentation, and a few other small errors, such as are very apt to creep into the first presentation of a method.

In the present paper it is our purpose to discuss in some detail various aspects of the Folin-Denis method for uric acid estima-

<sup>1</sup> O. Folin and A. B. Macallum, Jr.: this *Journal*, xiii, p. 363, 1912-13.

<sup>2</sup> O. Folin and W. Denis: *ibid.*, xiv, p. 95, 1913.

tion in urine and to describe certain proposed changes in the technique, which we believe represent distinct improvements.

Folin and Denis concluded that the reaction between the uric acid and the phosphotungstic acid reagent takes place in acid solution, and that the compound formed dissociates into the colored compound upon addition of the alkali. Such a behavior of uric acid (reduction in acid solution) would be so at variance with the general reducing properties of this compound that we were led to study this point a little further than was reported by Folin and Denis. These authors arrived at their conclusion that the reduction occurs in acid solution because the addition of alkali to the reagent and subsequent treatment of this mixture with uric acid failed to develop any color. We have found that this fact is explained by a decomposition of the reagent by the carbonate, and should not be taken to indicate a reduction in acid solution by uric acid. If the reagent be made alkaline with carbonate, then reacidified with phosphoric acid, added to the uric acid, and then carbonate added, no color develops. If, on the contrary, the carbonate be added to the uric acid and then the reagent be added, the full color develops. These experiments furnish direct evidence that the reduction occurs only in alkaline solution.<sup>3</sup> The point is obviously not of practical importance, and we mention it only to correct a possible misunderstanding regarding the reaction involved.

Another point in connection with the method is the question of the time required for the development of the maximal color. One would conclude from the Folin papers that the maximal color develops immediately on the addition of the sodium carbonate, and that, therefore, the results would not vary by waiting before adding the water. We have found that solutions which were diluted *immediately* after the addition of the sodium carbonate gave a reading about 3 mm. lower than those obtained after waiting forty to sixty seconds before diluting. In using the method this point should be taken into account, and one should wait one-half to one minute after adding the carbonate, before diluting with water. Longer waiting is to be avoided, because of a tendency to the development of turbidity if the solution stands too long before diluting.

The finding of a suitable standard solution for the uric acid comparison is obviously a most important point. As is well known, uric acid is readily soluble in alkalis, but in such solutions it rapidly undergoes decomposition or oxidation.

Folin and Macallum report that they devoted considerable time to trying to find a permanent standard. They found that colored glasses were not satisfactory as they transmitted too much or too little light; that some substances, including the aniline dyes, gave too bright a color to be used;

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<sup>3</sup> Some time ago Dr. Folin told one of us that he was well aware of this error in the early statement concerning the reaction and had intended to correct it subsequently, but had neglected to do so.

and that other substances gave blue solutions which were less bright and more greenish than the blue given by the uric acid. They stated that the uric acid reagent itself can be used as a standard by adding an excess of uric acid and standardizing against a lithium carbonate solution of uric acid, but Folin and Denis subsequently stated that this procedure was not satisfactory, because the color faded more rapidly than that obtained from solutions made with an excess of the reagent.

The standard which was first recommended by Folin and his collaborators was a solution of uric acid in lithium carbonate which was described as being made by washing 250 mgm. of Kahlbaum's uric acid into a flask with 25 to 50 cc. of water and adding 25 cc. of a 0.4 per cent lithium carbonate solution and shaking at intervals for an hour before diluting. In attempting to make up the lithium carbonate solution as directed, we have found that the uric acid fails to dissolve in the quantity of carbonate indicated within the course of an hour, or even much longer. We have sometimes started to prepare such a solution early in the afternoon, have shaken the mixture frequently for four hours and then left it standing over night, and still found an undissolved residue of uric acid the following morning. Even when obtained, the solutions of uric acid in lithium carbonate are not very stable. Folin and his collaborators stated that such a solution could be depended upon to remain unaltered for a week. This statement may hold for solutions kept cold, but certainly does not apply to solutions kept at room temperature in warm weather. Under such conditions we have noticed a 10 per cent loss of uric acid in two days, and a 25 per cent loss at the end of a week.

Folin and Denis finally suggested a permanent standard solution made by combining uric acid with formaldehyde. It is described as follows:

"One gram of uric acid in a volumetric liter flask is dissolved by means of an excess of lithium carbonate (200 cc. of a 0.4 per cent solution), to the solution is added 40 cc. of 40 per cent formaldehyde solution and the mixture is shaken and allowed to stand for a few minutes. The clear solution is acidified by the addition of 20 cc. of normal acetic acid and the whole is diluted up to the liter mark with water. The solution should remain perfectly clear and the next day (but not before) it can be standardized against a freshly prepared lithium carbonate solution of uric acid."

This formaldehyde-uric acid solution was recommended as meeting the requirements of a permanent standard and was said to act like ordinary pure uric acid solutions with reference to the quality and stability of the color produced with an excess of the reagent.

Contrary to the experience of Folin and Denis, we have not found the formaldehyde-uric acid solution which they propose to be a satisfactory standard, and we believe that very erroneous results may follow its use, unless the strictest precautions are taken as to standardizing the solution for exactly the temperature of the carbonate solution and diluting water employed for any individual determination. It is true that the formaldehyde-uric acid solution undergoes no deterioration upon standing, but the color yielded by a definite volume of the solution is affected by the tem-

perature of the carbonate solution and diluting water out of all proportion to the effect of these factors upon pure uric acid. This fact was first brought to our notice by finding a uric acid-formaldehyde solution on one day to give a value of 28.5 mm. for 5 cc. (compared with the color from 1 mgm. of uric acid set at 20 mm.), while the following day it read 24.5 mm. against the same quantity of uric acid. The temperature was the only variable factor involved, and this fact led us to try some experiments with the sodium carbonate solution and the diluting water at various temperatures, using the same carbonate solution and water for the pure uric acid as for the formaldehyde-uric acid solution.

To summarize our results in this connection it may be stated that the uric acid-formaldehyde solution showed variations in its colorimetric value of about 50 per cent when the temperature of the solutions used varied between 12 and 40 degrees. This temperature range is, of course, extreme, but so are the variations obtained. Other factors which affect the uric acid-formaldehyde solution besides the temperature are the samples of reagent used. (different samples of reagent prepared according to the same technique give very different color values), and the time elapsing before dilution.

As a result of our work on the formaldehyde-uric acid solution we were convinced that it is affected so differently from pure uric acid by many factors, that it is unreliable for use as a standard solution.

It was therefore desirable to find a suitable standard. Since temperature and time of standing before dilution affect pure uric acid as regards the quantity of color produced, we have worked altogether upon the assumption that the standard solution must be a solution of pure and unchanged uric acid. A standard solution which we employed for some time was one in which pyridin was used to effect solution of the uric acid. Although we have since found a more satisfactory solution, (and, we believe, an ideal standard solution), we shall describe the pyridin solution because it can be prepared very readily, is permanent for ten days under widely varying temperature conditions, and offers a convenient solution to check up another standard as occasion may require.

The pyridin-uric acid solution is made by washing 250 mgm. of uric acid (which should be powdered in a mortar before weighing) into an Erlenmeyer flask with 150 cc. of distilled water; then add 4 cc. of pyridin—Kahlbaum's is preferable—and warm the

mixture until clear (one-half minute to 65° to 70° is sufficient). Do not allow it to boil. Wash into a 250 cc. volumetric flask, cool, and dilute to the mark. One cc. of solution, of course, contains 1 mgm. of uric acid. As stated above, this solution loses no measurable amount of its color-yielding power within ten days, and is far more readily prepared, and more stable than the solution in lithium carbonate.

Later we were led to try the solubility of uric acid in phosphates. As a result of these trials we have found what would seem to be an ideal standard solution, in that it is very readily prepared, does not need to be standardized, and appears to keep indefinitely. This solution is prepared as follows: 9 grams of pure crystallized disodium hydrogen phosphate, together with 1 gram of crystallized sodium dihydrogen phosphate are dissolved in 200 to 300 cc. of hot water and the solution is filtered, if it was not previously perfectly clear. The filtrate is made up to a total volume of about 500 cc. with hot water, and this hot or warm (and perfectly clear) solution is poured upon exactly 200 mgm. of pure uric acid suspended in a few cc. of water in a liter volumetric flask. The mixture is agitated for a moment or two until the uric acid completely dissolves and is then cooled. Exactly 1.4 cc. of glacial acetic acid are added, and the contents of the flask are diluted to the mark and mixed. About 5 cc. of chloroform are then added to prevent growth of bacteria or moulds in the solution.

5 cc. of this solution contain exactly 1 mgm. of uric acid. We have had samples of the solution for periods of over two months now, and none has shown any detectable diminution of uric acid content as measured by its color-yielding power. The mixture is very delicately balanced. It reacts acid to litmus paper, but there is no tendency whatever to a separation of any uric acid. Yet if 0.5 cc. more of acetic acid per liter is added than is called for in the above directions an abundant crystallization of uric acid will take place within a few hours.

We cannot, of course, guarantee the *indefinite* stability of this standard solution, and until further tested it would best be checked up against a fresh uric acid solution (either in phosphate, pyridin, or lithium carbonate) once every month or two.

Before describing the exact technique which we suggest for

the determination of uric acid in urine we desire to call attention to one other general point. This concerns the concentration of the carbonate solution which is employed as the alkali. Folin and his collaborators have recommended in every instance the use of measured volumes of "saturated" sodium carbonate. Since the solubility of sodium carbonate varies enormously with the temperature, and since the concentration of carbonate markedly affects the quantity of color developed, we are of the opinion that it is best to use always a solution of sodium carbonate of known and definite concentration. We have found a 20 per cent solution (200 grams of carbonate dissolved in warm water and made up to 1 liter) of anhydrous sodium carbonate to be the most satisfactory. Such a solution is practically saturated at 20°, but can be cooled 5° or 10° lower for some hours without deposition of the salt. During cold weather we keep this carbonate solution in large Erlenmeyer flasks. If a deposition of crystalline carbonate occurs at any time the slightest warming of the solution for a moment or two will effect complete solution. Using this solution, one is certain of always employing the same amount of carbonate, and results are more uniform and satisfactory.

The method for the estimation of uric acid in urine proposed by Folin and Denis is as follows:

"From 1 to 2 cc. of urine are measured into an ordinary centrifuge tube by means of a modified Ostwald pipette. A sufficient amount of distilled water is then added to bring the volume of the liquid in the tube to about 5 cc., six drops of 3 per cent silver lactate solution, two drops of magnesia mixture, and a sufficient amount (10 to 20 drops) of concentrated ammonium hydrate to dissolve the silver chloride are then added. The tube is now centrifuged for one or two minutes, the supernatant liquid poured off, and to the residue in the bottom of the tube are added five or six drops of freshly prepared saturated hydrogen sulphide water, and one drop of concentrated hydrochloric acid, and the tube is placed in a beaker of boiling water until all excess of hydrogen sulphide has been driven off . . . . . When the tube has been cooled, add 2 cc. of the uric acid reagent, 10 cc. of saturated sodium carbonate solution, transfer to a 50 cc. volumetric flask and make up to volume. The color comparison is then made in the usual manner against the color obtained from 5 cc. of the standardized uric acid-formaldehyde solution (or a freshly prepared pure uric acid solution.)"

When properly carried out the above procedure of Folin and Denis yields accurate results. One point, however, has been omitted in their description, which is absolutely essential if results of any value are to be obtained. The point referred to is the necessity for thoroughly stirring up the pre-

precipitate containing the uric acid, after addition of the hydrogen sulphide water, so as to insure complete decomposition of the precipitate and liberation of the uric acid. If this simple step is omitted something over 50 per cent of the uric acid will be lost, whether the determination be applied to urine or to pure uric acid solution. The figures reported by Folin and Denis show that they must have adopted such a mechanical breaking up of the precipitate with the hydrogen sulphide water as a routine procedure, and simply neglected to mention it in the published description. It can be positively stated that results obtained by the Folin-Denis method without attention to the above mentioned point are totally worthless.

In the procedure for estimation of uric acid in urine which we wish to present we have combined the three solutions used in precipitating the uric acid into one, and have substituted a rapid and convenient procedure in place of the rather troublesome decomposition with freshly prepared hydrogen sulphide water. In this latter step we dissolve the precipitated uric acid in place of attempting to decompose it and precipitate the silver, as is done with hydrogen sulphide. The solvent employed is two drops of a 5 per cent solution of potassium cyanide. The cyanide instantly dissolves the precipitate and the silver exhibits no effect in any subsequent part of the procedure. While potassium cyanide has no power whatever of yielding any coloration with the uric acid reagent and alkali, still it exhibits a most interesting effect upon the color produced by a given quantity of uric acid. 1 mgm. of uric acid will give 18 per cent more color with the uric acid reagent and alkali, if previously treated with two drops of the cyanide solution, than is obtained when the cyanide is not present. This effect seems to be due chiefly to a marked diminution in the rate of the fading of the color from solutions containing the cyanide. Other factors may also be involved and we expect to study this point further. For practical purposes it will be sufficient to point out that the final results are unaltered providing the two drops of cyanide are added to both the standard solution and the unknown. The slower fading of the color under these conditions is a distinct advantage. If the cyanide solution be kept in a small dropping bottle the addition of two drops to the standard does not require a moment of time.

The modified technique which we wish to recommend for the determination of uric acid in urine is as follows:

Such a volume of urine as will contain from 0.7 to 1.3 mgm. of uric acid (2 to 4 cc. is usually the right amount) is measured into



a centrifuge tube, diluted to about 5 cc. with water, and treated with 15 to 20 drops of an ammoniacal silver magnesium solution.<sup>4</sup> The contents of the tube are now mixed with a small stirring rod and the tube is then centrifuged for one or two minutes. The supernatant solution is then poured off *as completely as possible*, the tube being inverted, and the inside of the lip touched with a towel or piece of filter paper.<sup>5</sup> The residue in the tube is then treated with two drops of 5 per cent potassium cyanide solution,<sup>6</sup> and the mixture thoroughly stirred with a narrow stirring rod for half a minute. A few drops (0.5 to 1.0 cc.) of water are then added and the solution is again stirred.<sup>7</sup> 2 cc. of the uric acid reagent<sup>8</sup> are then added and the mixture is stirred, after which

<sup>4</sup> This solution has the following composition:

	cc.
3 per cent silver lactate solution .....	70
Magnesia mixture.....	30
Concentrated aqueous ammonia.....	100

Shortly after being mixed the solution will develop a slight turbidity, which should be filtered off. The clear filtrate will keep indefinitely.

The magnesia mixture is made as follows: dissolve 17.5 gm. of crystallized magnesium sulphate and 35 gm. of ammonium chloride in about 100 cc. of water; add 60 cc. of concentrated aqueous ammonia, and dilute to 200 cc.

<sup>5</sup> Failure to pour off the supernatant fluid completely is apt to be followed by rapid development of turbidity in the final colored solution, owing to the presence of appreciable quantities of ammonia. Where directions are followed exactly as given in this paper, we have never encountered any trouble from turbid solutions. Should turbidity develop, filtration can be resorted to, but in such cases the standard solution must also be filtered into the colorimeter chamber. This is due to the fact that reoxidation of the colored compound is accelerated by filtration, and an unfiltered clear standard will read higher than the same solution after filtration.

<sup>6</sup> Potassium cyanide solutions will keep for several months, but not for years. Old solutions which have become colored or which smell strongly of ammonia should not be employed.

<sup>7</sup> At this point a perfectly clear solution is obtained where pure uric acid solution in carbonate or pyridin is used. In the case of urine some magnesium ammonium phosphate is precipitated (together with the uric acid) which does not dissolve in the cyanide solution. After adding the two subsequent reagents, however, a perfectly clear solution is obtained.

<sup>8</sup> The uric acid reagent of Folin and Denis is prepared by boiling together 100 gm. of sodium tungstate, 80 cc. of 85 per cent phosphoric acid, and 750 cc. of water under a reflux condenser for an hour and a half, cooling the solution, and diluting to one liter.

10 cc. of 20 per cent sodium carbonate solution are added, the mixture is washed quantitatively into a 50 cc. flask at the end of about one-half minute, and diluted to the mark. This solution is compared in a colorimeter with a simultaneously prepared colored solution obtained by treating 5 cc. of the standard uric acid solution (described earlier in this paper) contained in a 50 cc. flask with two drops of the potassium cyanide solution, 2 cc. of the uric acid reagent, 10 cc. of 20 per cent sodium carbonate solution, and diluting to the mark at the end of about one-half minute. The standard solution is best set at a height of 15 mm. in the colorimeter.

The procedure described above yields quantitative results for pure uric acid solutions and for uric acid added to urine. The figures obtained for uric acid in various samples of urine agree within a few per cent with those obtained by the Folin-Shaffer method. We are inclined to regard the new procedure as perhaps more accurate than the titration method of Folin and Shaffer.



## ON THE COLORIMETRIC DETERMINATION OF URIC ACID IN BLOOD.

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(Received for publication, February 8, 1915.)

While the quantitative determination of uric acid in blood has been attempted frequently for some years, it seems safe to say that the first figures reported in connection with such determinations which have any real quantitative value were those offered by Folin and Denis.<sup>1</sup> These investigators made use of a method devised by themselves which (for fresh blood at least) is quite accurate. There are, however, a few steps in the process where there is room for improvement. In working with the Folin-Denis method for uric acid in blood one cannot but be impressed with the fact that the final steps in the process are far from ideal. The relatively large amount of protein which escapes precipitation during the preliminary coagulation with dilute acetic acid is a distinct hindrance to accurate and satisfactory work later on. For this reason the present writer has adopted a second precipitation of protein (by means of colloidal iron) which gives perfectly clean solutions for the final precipitation of the uric acid. In addition to this point, certain other modifications in the procedure have been adopted in accordance with the suggestions contained in the preceding paper upon the estimation of uric acid in urine.

The proposed modified procedure for the estimation of uric acid in blood is as follows:

20 cc.<sup>2</sup> of blood are added to 100 cc. of boiling 0.01 N acetic

<sup>1</sup> O. Folin and W. Denis: this *Journal*, xiii, p. 469, 1912-13; xiv, p. 29, 1913.

<sup>2</sup> Smaller volumes of blood may be employed, and the quantity of acetic acid and of water correspondingly reduced. In the rest of the process the procedure should not be materially modified. Unless the quantity of uric acid present is very large, the results are far more accurate where 20 cc. of blood are used.

### 630 Colorimetric Determination of Uric Acid

acid<sup>3</sup> in a casserole and the mixture is heated to boiling for a moment. The casserole is then removed from the flame and 200 cc. of boiling distilled water are added. The mixture is then poured upon a folded filter, and the residue washed with 50 cc. of boiling water (heated in the same casserole in which the original coagulation took place). The total filtrate is now transferred to a casserole and boiled rapidly down to a volume of about 25 cc. This solution is poured into a small flask roughly marked to indicate a volume of 50 cc. The contents of the casserole are washed quantitatively into the flask with the help of two or three portions of water, heating the water to vigorous boiling, and rubbing the sides of the casserole with a rubber-tipped stirring rod each time. The total volume in the flask should not exceed 50 cc. after addition of the washings. The turbid solution in the flask is now thoroughly cooled under running water and 2 cc.<sup>4</sup> of colloidal iron solution (Merek's "Dialyzed Iron," 5 per cent solution) are added while the flask is gently rotated. The mixture is then filtered through a small folded filter into a 100 cc. Jena Florence flask, the residue on the filter being twice washed with distilled water. The filtrate obtained here should be as clear and colorless as distilled water. The solution is now boiled down to a volume of 1 to 2 cc., (care being taken during the early stage of the process to avoid bumping), then carefully poured into a small centrifuge tube, and the flask washed out with three portions of water (1 to 2 cc. each), heating each to boiling in the flask and shaking thoroughly prior to transferring it to the centrifuge tube. The contents of the tube (which should have a volume of 5 to 10 cc.) are now cooled and treated with twenty drops of the ammoniacal silver magnesium solution described in the preceding paper. The contents of the tube are mixed thoroughly with the help of a narrow stirring rod, and the tube is then cen-

<sup>3</sup> This is the same concentration of acetic acid as employed by Folin and Denis. It works well for fresh blood. In the case of old samples of blood it is often more satisfactory to employ acetic acid of half this concentration.

<sup>4</sup> With old samples of blood it may be necessary to add 3 or 4 cc. of the iron solution and a little 10 per cent sodium chloride solution. When the precipitate separates in large flocculent masses the right quantity of iron has been added. Any excess of iron must be avoided, as it would oxidize some of the uric acid later on in the process.

trifuged for one or two minutes. The supernatant fluid is then poured off *as completely as possible*, and to the residue in the tube are added one or two drops of 5 per cent potassium cyanide solution,<sup>5</sup> and the mixture is thoroughly stirred for a moment. A few drops of water are then added and the mixture is again stirred. 1 or 2 cc. of the uric acid reagent of Folin and Denis are then added (1 cc. if the bulk of the original precipitate was very small, otherwise 2 cc.), and, correspondingly, 5 or 10 cc. of 20 per cent sodium carbonate solution are then added, and the colored solution is washed quantitatively into a 25 or 50 cc. flask and diluted to the mark with water. This solution is then compared in a colorimeter with the colored solution obtained by treating either 0.5 or 1 mgm. of uric acid<sup>6</sup> with 2 cc. of the uric acid reagent, 10 cc. of 20 per cent sodium carbonate solution, and diluting to 50 cc. at the end of about one-half minute.

In conclusion it may be stated that this method appears to give the "free" uric acid in blood.<sup>7</sup> The results obtained for the most part closely parallel those obtained by the Folin-Denis method. There is no precipitation of the uric acid in blood by colloidal iron. The marked advantage gained by this precipitation is that the final solutions obtained are practically never turbid, and the color matches that of the standard solution exactly. In the Folin-Denis process the addition of the ammoniacal silver and magnesium solutions is followed by the precipitation of a quantity of protein in addition to the uric acid. In the present method this is not the case, and with a little practice one can tell from the precipitate obtained when the silver solution is added, what standard solution should be employed for the final comparison.

<sup>5</sup> If the bulk of the precipitate is very small one drop of the cyanide solution should be added, and the colored solution ultimately obtained should be diluted to only 25 cc. If the bulk of the precipitate indicates a larger amount of uric acid, two drops of cyanide solution are added and the final dilution is made to 50 cc.

<sup>6</sup> For preparation of standard uric acid solutions, see the preceding paper.

<sup>7</sup> The point involved here is discussed in the following paper.



**STUDIES IN URIC ACID METABOLISM. I.  
ON THE URIC ACID IN OX AND IN CHICKEN BLOOD.**

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(Received for publication, February 8, 1915.)

As preliminary to certain investigations in uric acid metabolism it was thought desirable to try out the Folin-Denis method for estimating uric acid in blood. Ox blood was selected as a basis for this work, on account of its ready availability in large quantities. During the investigation certain facts have been developed which would appear to be of considerable interest in connection with intermediary purine metabolism, especially that of uric acid. The present communication is in a way preliminary in nature, since many obvious problems which the data presented bring up cannot be reported upon in this paper. But as concerns the data offered, it may be stated that they have been so repeatedly verified that they seem to admit of no question.

As far as the writer is aware, the first and only recorded analysis for the uric acid content of ox blood is that reported by Folin and Denis<sup>1</sup> in a table covering the uric acid content of the blood of several species; wherein ox blood "Beef (mixed blood)" is given as containing 0.2 mgm. of uric acid per 100 gm. of blood.

Employing the method described in the preceding paper the writer has found an average value of 0.5 mgm. of uric acid per 100 cc. of defibrinated fresh ox blood. This figure is based upon an analysis of seven samples of mixed blood. The highest value obtained was 0.67 mgm., the lowest 0.41 mgm., per 100 cc. of blood. There is no apparent explanation of the discrepancy between the present values reported and that of Folin and Denis. Using substantially the Folin-Denis method, the present writer

<sup>1</sup> O. Folin and W. Denis: this *Journal*, xiv, p. 29, 1913.



has obtained results only very slightly lower than those obtained by the modified procedure. In the blood of other species so far examined we have been able to duplicate more closely the figures reported by Folin and Denis.

In order to determine how much "interfering" substance there might be in ox blood as regards determining the uric acid without any preliminary precipitation with silver, some determinations were made omitting this step. In such instances the blood was coagulated, filtered, and washed as described in the preceding paper, the filtrate boiled down, cooled, precipitated with colloidal iron, and the filtrate from this precipitation boiled down to a small volume, and this total solution treated directly with the uric acid reagent and the alkali. These observations showed the total color-yielding power of the blood to be equal to about 4 mgm. of uric acid per 100 cc. of blood, or about 800 per cent of the uric acid actually found. A similar procedure with chicken blood gave a total color-yielding power relatively only slightly in excess (20 per cent) of the uric acid actually found. It seemed so questionable whether phenols or other interfering substances should exist in ox blood in such relatively enormous amounts that the matter was subjected to further investigation.

The method of study used was based upon the assumption that a part of the uric acid in ox blood might exist in combination, and so escape precipitation by the silver magnesium solution.

Some experiments were accordingly tried in which the filtrate obtained after precipitation of the last traces of protein with colloidal iron was boiled down after addition of some concentrated hydrochloric acid. The following experiment may be cited in this connection.

20 cc. of fresh defibrinated ox blood, treated according to the modified process for uric acid estimation described in the preceding paper, showed a uric acid content of 0.56 mgm. per 100 cc. A second 20 cc. portion of the same blood was treated in the same way, except that 2 cc. of concentrated hydrochloric acid were added to the filtrate after the colloidal iron precipitation. After boiling down to a small volume the solution was precipitated with the silver magnesium solution as in the other instance, except that an excess of strong ammonia was added to neutralize the marked acidity of the final solution. The treatment of the precipitate obtained here was identical with that of the preceding sample. The uric acid obtained in this instance amounted to 5.87 mgm. per 100 cc. of blood, or about 1100

per cent of that obtained when the boiling with hydrochloric acid was not employed. This result has been confirmed by more than twenty-five similar comparative determinations upon about fifteen different samples of ox blood. In every instance at least 1000 per cent more uric acid was obtained after the hydrolysis with hydrochloric acid than without such hydrolysis. The greatest quantity obtained after the hydrolysis was 7.2 mgm. of uric acid per 100 gm. of blood, or about thirty-six times as much as was reported by Folin and Denis for this blood, and about half as much again as these investigators found in chicken blood. The quantity found in the instance cited above was about 1300 per cent of the uric acid found in the same sample of blood without the final acid hydrolysis.

Up to the time of finding the indication of a "combined" uric acid in ox blood, all the writer's work was carried out upon fresh samples of blood, upon the assumption that uric acid would probably disappear rapidly through oxidation or decomposition. After the presence of combined uric acid was demonstrated, however, it seemed possible that the blood might contain an enzyme capable of splitting this compound, and samples of blood were examined for the uric acid content every day or two for periods of about two weeks. Toluene and chloroform were used as preservatives, and the blood was left at room temperature. The results obtained in this connection have shown that, upon standing, ox blood shows a marked and rapid increase in the "free" uric acid, with a corresponding decrease in the "combined" uric acid. The following figures are offered as illustrative. A sample of fresh ox blood showed a "free" uric acid content of 0.50 mgm. per 100 cc., and a "total" uric acid content (*i.e.*, after hydrolysis of the colloidal iron filtrate) of 6.7 mgm. per 100 cc. Two days later this same blood showed a "free" uric acid content of 2.02 mgm. per 100 cc., while the "total" uric acid content remained unchanged. Two days later the "free" uric acid content had risen to 4.15 mgm. per 100 cc. Four days later this figure had risen to 5.8 mgm. When the blood was two weeks old it showed a "free" uric acid content of 7.5 mgm. per 100 cc., and no longer showed any trace of "combined" uric acid. That the liberation of free uric acid from the combined substance was not due to a spontaneous decomposition of the latter is shown by the fact that solutions prepared by coagulation and filtration from the original fresh blood, and kept for the same length of time with the same preservative, showed no change in the ratio between the free and

combined uric acid. We may therefore postulate an enzyme action in the blood leading to the increase in the "free" uric acid.

It will be noted that the final figure (7.5 mgm. per 100 cc. of blood) for the uric acid at the end of two weeks is quite appreciably higher than that obtained by the acid hydrolysis method upon the fresh blood, where a value of 6.7 mgm. of uric acid per 100 cc. of blood was obtained. We may say that this is almost invariably the case. After standing for some time the blood will tend to show a little more uric acid than can be demonstrated by the acid hydrolysis method as "total" uric acid in the fresh blood. This brings up the question whether there is really some formation of new uric acid while the blood stands, or whether the acid hydrolysis method fails to give an absolutely quantitative yield of free uric acid from the combined substance. The following experiment serves to show that it is probably not possible to secure a concentration of hydrochloric acid for the hydrolysis which will quantitatively split the uric acid compound without destroying some of the uric acid itself. The experiment also serves to show that in the fresh blood we are dealing with a relatively very stable combination of the uric acid.

A sample of fresh blood which yielded 0.46 mgm. of "free" uric acid per 100 cc. of blood was employed, and samples of the filtrate from the colloidal iron precipitation were boiled down with varying concentrations of hydrochloric acid, prior to the precipitation with the silver magnesium reagent. The results were as follows: with 2 cc. *N* HCl, 0.88 mgm. of uric acid per 100 cc. of blood; with 1 cc. concentrated HCl, 3.75 mgm.; with 2 cc. concentrated HCl, 5.68 mgm.; with 3 cc. concentrated HCl, 5.72 mgm.; with 5 cc. concentrated HCl, 5.2 mgm.; with 10 cc. concentrated HCl, and boiling for half an hour prior to concentrating, 4.25 mgm. After standing for two weeks this same blood showed a free uric acid content of 6.4 mgm. per 100 cc.

It would thus appear that the uric acid combination is so stable that for its quantitative splitting one must increase the acid concentration to a point where some of the uric acid itself suffers decomposition. One can, however, count upon obtaining at least 90 per cent of the total uric acid present in ox blood through the use of 2 cc. of concentrated hydrochloric acid for the hydrolysis.

Such a figure as 7.5 mgm. of uric acid per 100 cc. of blood for an animal which eliminates almost no uric acid in the urine was

considered so surprising that it was believed that such a figure could not be accepted without the final corroboration of isolating and identifying the uric acid. If the uric acid really exists in the quantities indicated, its isolation and identification should not be at all a difficult matter. The following experiment (one of three in this connection which all gave similar results) is cited to show that the substance above assumed to be uric acid is indeed that compound.

100 cc. of whole defibrinated ox blood were added to 500 cc. of boiling 0.01 N acetic acid and the mixture was heated to boiling. One liter of boiling water was added and the mixture filtered, the residue being washed with 100 cc. of boiling water. The total filtrate was boiled down to a volume of about 125 cc., and this solution washed quantitatively into a flask with the help of boiling water. The total volume of the solution was then about 175 cc. The contents of the flask were then cooled under running water, and 10 cc. of 5 per cent colloidal iron solution were added, followed by 10 cc. of 10 per cent sodium chloride solution. The mixture was then filtered, the residue being thoroughly washed with cold water. The total filtrate was then treated with 10 cc. of concentrated hydrochloric acid and boiled down to a volume of about 10 cc. This solution was washed quantitatively into a centrifuge tube with the help of 10 cc. of hot water. The solution in the centrifuge tube was then cooled and treated with 5 cc. of the ammoniacal silver magnesium solution and stirred, while cooled under running water,<sup>2</sup> and an excess (3 cc.) of strong ammonia added to dissolve the silver chloride. The solution was then again cooled and stirred, and the tube was centrifuged for about two minutes. The supernatant fluid was then poured off as completely as possible and the precipitate in the bottom of the tube was thoroughly stirred up with 5 cc. of water. 5 cc. of freshly saturated hydrogen sulphide water were then added, and the mixture was thoroughly stirred for a few minutes. The mixture was then washed into a beaker and the hydrogen sulphide removed by boiling, and the silver sulphide by filtration. The filtrate was boiled down to a small volume and finally taken to dryness on a water bath. The residue was taken up in 5 cc. of boiling water, washed into a centrifuge tube, treated with 5 cc. of the ammoniacal silver magnesium solution, and again centrifuged. The precipitate was treated with water and hydrogen sulphide solution as before, boiled, filtered, and taken down to dryness. The residue was then treated with 5 cc. of boiling water, 1 cc. of glacial acetic acid added drop by drop, and the mixture allowed to cool spontan-

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<sup>2</sup> Whenever the acid hydrolysis has been employed it is best to cool the solution during the addition of the silver solution and of the strong ammonia. Unless this precaution is observed traces of the uric acid may be oxidized by the silver.

cously. Uric acid separated in typical colorless crystals. These were washed carefully into a dry, weighed centrifuge tube and the tube was centrifuged for two minutes. The crystals were washed with a little water and then with alcohol (by stirring with the liquid and then centrifuging), the fluid was poured off, and the tube dried to constant weight at 90°. The crystals thus obtained weighed 6.7 mgm. They were treated with 3 cc. of 0.4 per cent lithium carbonate solution, in which they readily dissolved. The solution thus obtained was diluted to exactly 10 cc. 1 cc. of this solution, when treated with 2 cc. of the uric acid reagent, 10 cc. of carbonate solution, and diluted to a measured volume, gave a color value corresponding to 6.65 mgm. of uric acid for the 10 cc. of solution, as against 6.7 mgm. found by weight. A second portion of the lithium carbonate solution was used for the murexide test. The reaction was positive. A third portion (5 cc.) of the lithium carbonate solution was used for a nitrogen determination. 1.05 mgm. of nitrogen was found, against 1.11 mgm. calculated for uric acid.

Thus 6.7 mgm. of uric acid were isolated from 100 cc. of ox blood, and identified by crystalline appearance, solubility, murexide test, colorimetric value, and nitrogen content. The regular colorimetric determination (as employed throughout this work) gave a value of 7.0 mgm. of "total" uric acid per 100 cc. for this same blood, thus showing the accuracy of the colorimetric process, and of the procedure used for isolation of the uric acid.

The final point which can be reported upon at present concerning the uric acid in ox blood is in relation to the question of the distribution of the uric acid between the corpuscles and the serum. In this study clear serum obtained from fresh blood by the use of the centrifuge, and (unwashed) corpuscles obtained in a similar manner have been used. The results obtained are so clear cut that no detailed description of the procedures employed is necessary. The serum and corpuscles (separately) were coagulated in boiling 0.01 N acetic acid, and treated just as reported for whole blood, using the hydrochloric acid hydrolysis. Thus the "total" uric acid present was determined. *The results showed that the uric acid is quantitatively contained in the corpuscles.* With 25 cc. of clear serum, not the slightest color reaction for uric acid could be obtained with the trace of silver precipitate ultimately obtained. Perhaps larger quantities of the serum would reveal traces of uric acid, but certainly not more is present than could well be accounted for by the few disintegrated corpuscles. The corpuscles, on the contrary, yielded figures for uric acid closely

approximating those obtained for the volume of the whole blood from which they were derived.

Sharply contrasted with the findings for the uric acid contained in ox blood stand the results obtained for chicken blood. Folin and Denis reported for the blood of this species a content of 4.8 mgm. of uric acid per 100 gm. of blood. During the present work we have been able to corroborate this finding. By the use of the methods applied to ox blood it has not been possible to demonstrate the presence of any combined uric acid in chicken blood. There is no increase whatever in the uric acid obtained after the acid hydrolysis, nor does the uric acid content of the blood show any increase upon standing. Furthermore, while the serum of ox blood is (as far as is demonstrable) free from uric acid, the uric acid in chicken blood is almost wholly contained in the serum. The concentration in the serum is very appreciably greater than in the whole blood. Thus a sample of chicken blood which contained 4.8 mgm. of uric acid per 100 cc. of whole blood, showed a concentration of 5.7 mgm. per 100 cc. of serum. The corpuscles obtained from this blood (washed once with 0.7 per cent sodium chloride solution) had a uric acid content of only 0.36 mgm. per 100 cc. of whole blood. Probably a more complete removal of the serum would result in still lower figures.

Thus it will be seen that ox blood contains about 50 per cent more uric acid than does chicken blood. In ox blood the uric acid is (for the most part, if not wholly) in combination and within the corpuscles, while in chicken blood the uric acid circulates free and is contained in the serum.

It would not seem desirable to go into much theorizing as a result of the data presented in the present paper. Perhaps one tentative hypothesis would be that uric acid which can be further catabolized exists in combination in the organism, while that which is to be eliminated as such circulates in the blood in the free state. In subsequent communications the theoretical aspects of the subject will be taken up in some detail. Concerning the nature of the combination in which the uric acid exists, little more can be stated at present than that it is certainly not protein in nature, since it is to be found in the filtrates after protein has been removed as completely as possible.

The work suggested by the results recorded in the present

paper is being continued. The question as to which form of corpuscle contains the uric acid in ox blood is of obvious importance, and will be reported upon shortly. We are already studying the blood of other species, including normal and pathological human blood (especially that in gout). It would appear that the data already presented may have important bearing upon studies of uric acid destruction by tissues and various organs in different species.

## NUTRITION WITH PURIFIED FOOD SUBSTANCES.<sup>1</sup>

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For many years sporadic attempts have been made to nourish animals on mixtures of purified food substances, but with relatively little success. The importance of knowing just what complexes may arise through the synthetic activity of the cells, and what must of necessity be supplied in the diet has stimulated investigators to attain this end. The early workers attributed the failure of their animals to grow on such rations to various causes, among which the most common was the inability of the animal cell to synthesize the constituent parts of the nucleic acid complex and hemoglobin from the organic complexes of such a diet. Much of the discussion was directed to the need of the higher animals for organic phosphorus compounds, such as lecithin, nucleo- and phospho-proteins. The addition of these substances to rations of purified foodstuffs, however, did not lead to better results.

The reasons assigned for the early failures have now been entirely set aside. We know that these complexes containing phosphorus, and also the purines and pyrimidines, must be produced synthetically from inorganic phosphates and from the nitrogen derived from purified protein.<sup>2</sup>

With diets of purified casein, dextrin, milk sugar, agar-agar, and salts from reagents (a fat-free ration), rats may grow well during three or four months. The period of growth depends

<sup>1</sup> Published with the permission of the Director of the Wisconsin Experiment Station.

<sup>2</sup> E. V. McCollum: *Am. Jour. Physiol.*, xxv, p. 120, 1909-10. A review of the older literature relating to the feeding of purified foodstuffs is given here. See also E. V. McCollum, J. G. Halpin, and A. H. Drescher: *this Journal*, xiii, p. 219, 1912-13. T. B. Osborne and L. B. Mendel: *Carnegie Institution of Washington, Publication No. 156*, pts. i and ii, 1911.



upon the natural stamina of the individual. Many make no growth at all; others, and indeed the greater number, will make about half the normal growth, while a few individuals of exceptional vitality will grow at the normal rate. By careful attention to the selection for our breeding stock during several generations we have greatly increased the percentage of individuals which approximate the performance illustrated by Charts I and II. The domestic rat has been kept under conditions where inferior specimens have survived and propagated until the general vitality of the strain is low. This fact, however, does not detract from the value of our observations with rats of improved vitality. What we are seeking to learn is the chemical powers of the cells of the mammalian organism, and in order to do this we must eliminate just as far as possible all other factors which can influence the animal's tendency to grow. The power of growth is not the same in all individuals of the same species, or even of the same family, yet we must admit that the stronger ones are the more normal, in that all their physiological processes are functioning with greatest smoothness and power. In nutrition studies it is of the greatest importance that success or failure shall turn upon a single factor. Obviously where animals with the most exceptional vigor are not used, even though a considerable number be employed, the conclusion from the experiments may be entirely misleading. If success in growth or reproduction is to turn on the single factor of the lack of a certain complex or complexes on the ration, we must know that our animals are so vigorous that they are accomplishing everything which any individual of the species is prepared to accomplish, as far as the chemical character of the ration will admit.

Osborne and Mendel,<sup>3</sup> as well as we, have succeeded in obtaining these results with rations of the same general make-up as that described above. At one time they were convinced that a normal growth curve in the rat, covering a period of sixty days, was evidence enough to disprove the contention of Stepp that certain fats or substances soluble in fat solvents were essential for the maintenance or growth of an animal.<sup>4</sup> Four months

<sup>3</sup> Osborne and Mendel: *loc. cit.*, pt. ii; this *Journal*, xii, p. 81, 1912.

<sup>4</sup> W. Stepp: *Biochem. Ztschr.*, xxii, p. 452, 1909; *Ztschr. f. Biol.*, lvii, p. 135, 1912.

after we published our observation that pure butter-fat or egg-fat had a most remarkable influence in causing a resumption of growth in rats which were brought to a condition in which further growth was impossible on a fat-free ration, they published data which confirmed our own results in every respect and pointed out that commercial cod liver oil had a similar property. It is now certain that there is a group of fats, which includes those mentioned above, which can be differentiated in their biological value from another group which includes olive oil, cottonseed oil, and almond oil, and apparently also the body fats of animals, as lard or tallow. We are not ready, however, to pronounce finally on the latter two.

At the present time, all the published records obtained with purified food substances, even with butter-fat or other fats having similar biological value, fall far short of complete nutrition. The longest growth curves described by Osborne and Mendel which were obtained with their "artificial protein-free milk" cover about 120 days. Their continued use of "natural protein-free milk" would lead to the assumption that they still attributed to this product a peculiar value not possessed by the imitation which they were able to prepare from salts and milk sugar.

The widely discussed differences in the nutritive value of fresh foods as compared with the same substances heated or chemically manipulated, and of "natural protein-free milk" as compared with the "artificial" product postulate the existence of substances of unknown nature other than those carried by certain fats, which are essential constituents of the diet, and without which such pathological conditions as polyneurites, scurvy, etc., result. It is as a contribution to our knowledge on this particular point that the present paper is offered.

In Charts I to IX are shown the curves of groups of rats whose diets differed only in respect to the presence or absence of fats, or as to the character of the fats contained. It is evident from these curves that the addition of commercial olive oil (Chart IV) or cottonseed oil (Chart III) had no effect in changing the general shape of the curve of growth or delaying the time of onset of failure of nutrition. In marked contrast to these two, however, is the result of adding the ether extract of dried, ripe

cod testicle to the ration (Chart VI). A similar effect is apparent in Chart VII where the ether extract of pig kidney was added. Both these fat mixtures promote growth, as does butter-fat.

Attention should be called to the small amount of growth which the rats in Charts VI and VII made while on a fat-free diet in the early weeks of their record, as compared with that of the rats in Charts I and II. The former were animals of decidedly lower vitality and the ration had to be improved in its growth-promoting properties before they were able to grow. Considering this difference in vitality the effects of the fats of the cod testicle and of pig kidney appear very efficient in producing the effects produced by butter-fat, egg-fat, and commercial cod liver oil<sup>5</sup> with respect to growth.

An inspection of Chart VIII shows how nearly complete is the nutrition of rats fed the same ration of purified food substances employed throughout this series of experiments, but with the addition of butter-fat to the extent of 5 per cent of the food mixture. Two of the females, whose curves are shown, have made perfectly normal growth to the normal adult size and have successfully brought forth and nourished two and three litters of young, respectively, on this food. Some of these young have successfully grown to a weight of 190 grams on the mother's diet and are at this writing in an apparently perfect state of nutrition.

In marked contrast to the performance of these rats is the failure of those rats which received 5 per cent olive oil (Chart IV) instead of butter-fat, to produce any young, although they were kept with males all the time. In our experience normal growth to the normal adult size, at about the usual rate, is not satisfactory evidence of perfect nutrition. Only when there is normal reproduction and suckling of the young, and repetition of this at the normal intervals, can nutrition be said to be entirely satisfactory.

We would call attention here to the conditions under which our rats have been kept. The cages are two feet square and twenty inches high, made of wire netting nailed to a wooden frame. The entire floor space is covered by a square pan of galvanized

<sup>5</sup> Osborne and Mendel: this *Journal*, xvii, p. 401, 1914.

iron which fits snugly. In the pan clean wood shavings cover the bottom. The food and water supply is suspended so that it does not become mixed with shavings. A good opportunity is thus afforded for ventilation and exercise. Several rats are always kept together.

The rats ate a certain amount of wood, but this can hardly be assumed to be a factor in the nutrition of the animals in any way except, possibly, as a favorable physical stimulant. Analyses have shown it to contain approximately 0.2 per cent of nitrogen, and since the small bits of wood are not disintegrated at all it is hardly to be supposed that an appreciable amount of nitrogen could be absorbed from this source. We do not look upon the consumption of a small amount of wood fiber as objectionable to any greater degree in this type of experiment than is the feeding of agar-agar. We point out this fact of wood consumption in order that all possible objections to the technique may be known. All lots of our rats whose curves are discussed in this paper had the same environment.

It should also be pointed out that we fully appreciate the possible valid objection to the use of milk sugar in a ration in which purity of the constituents is sought. We have employed both Merck's and Kahlbaum's lactose in about equal amounts in all the work here reported. While this lactose showed nitrogen contents varying from 0.02 per cent to 0.034 per cent, which would make the nitrogen consumption from the source seem negligible, we cannot look upon lactose of even this degree of freedom from nitrogen as a pure sugar for this work. Experiments which are now in progress will determine whether this ration loses any of its power of sustaining growth or reproduction through further purification of the lactose, or strongly heating the latter. Until such inquiry is complete we shall avoid a discussion as to the necessity of vitamins or hormones in the diet.

Throughout these experiments distilled water only was supplied. The dextrin was prepared from high grade corn starch, moistened with 0.5 per cent citric acid solution, and heated at fifteen pounds' pressure in an autoclave during three hours. It was then dried and ground. The butter-fat, of course, can not be looked upon as a pure substance, except in so far as it consisted only of that portion of butter which was soluble in ether.

Iodine was supplied in the drinking water once each week.

With the reservations noted above in regard to the wood fiber and lactose this ration may be safely stated to be made up of purified foodstuffs. The casein showed only about 0.03 per cent of calcium oxide as an average of several analyses. This should serve as convincing evidence that no other impurities were present in appreciable amounts. *It seems safe to say that we have here attained an unprecedented success in the nutrition of rats with rations of a higher degree of purity of each constituent than any yet reported.*

Any discussion of nutrition with purified foodstuffs at the present time should include a consideration of the use of "protein-free milk" which has been employed during more than two years in the extensive experiments of Osborne and Mendel.<sup>6</sup> This product obtained by coagulating the albumin from acidified whey and evaporating the filtrate, contains, according to their analyses, about 0.7 per cent of nitrogen. This we can confirm as the result of a number of analyses of samples prepared by us. This amount of nitrogen, if it represents protein, would make 4.37 per cent of the total material. Of this product they employ 28 per cent in the ration, which would mean 1.22 per cent of protein added to the diet from milk. They offer no biological tests for the nutritive value of this nitrogen, or lack of the same, but instead they substitute a mathematical treatment of the values obtained by Munk, published twenty years ago, of the amount of protein and non-protein nitrogen present in milk, based upon precipitation of "protein" with reagents. In this way they arrive at the conclusion that their food pastes, made up with this ingredient, contain only 0.48 per cent of milk protein. At the same time they fix the lowest plane of protein intake essential for even slight growth at 7 to 9 per cent of the food mixture.<sup>7</sup> By this process they conclude that the amount of protein added to their rations in the form of protein-free milk is too small to constitute an important factor, and they assume that the experimental data obtained with their rations is comparable with that of other workers who have adhered to the

<sup>6</sup> Osborne and Mendel: *Carnegie Institution of Washington, Publication No. 156*, pt. ii, p. 81, 1911.

<sup>7</sup> Osborne and Mendel: *Ztschr. f. physiol. Chem.*, lxxx, p. 348, 1912.

use of mixtures of more or less pure substances in their efforts to solve the problems which can be solved only through such methods.

In a previous paper<sup>8</sup> we have pointed out that they assume entirely too high a figure for the plane of intake of protein (7 to 9 per cent) which will serve to maintain young rats in body weight. This we have fixed at about 3 per cent for milk proteins. There is contained in all the rations fed by Osborne and Mendel, therefore, at least 41 per cent of the amount of milk nitrogen necessary to maintain a young rat in body weight, provided the hydrogen is in the form of protein or cleavage products of milk protein which are its equivalent. It is a well known fact that proteolytic ferments are present in milk, and this fact alone should make us cautious about accepting the results of precipitating "protein" from milk as an index to the nutritive value of the part of the nitrogen not precipitated by protein reagents. It is true that qualitatively nearly all the constituents of blood have been identified in milk, but these are present in extremely small amounts.

That the nitrogen of protein-free milk is nearly, if not quite, equivalent to milk protein nitrogen as a nutrient for young rats is made evident from experiments which are at present in progress in our laboratory, in which young rats have maintained their body weight during eighty days on a mixture of milk powder protein 1.5 per cent, protein-free milk protein ( $N \times 6.25$ ) 1.5 per cent, with dextrin and butter-fat to make 100. We have already mentioned<sup>9</sup> that milk protein in the form of skim milk powder fed at levels of 2 per cent of the food mixture causes steady loss of weight, while 3 per cent of the same proteins serves to maintain body weight in rats for periods beyond 100 days. It is evident that the total nitrogen of protein-free milk serves as nearly, if not quite, the equivalent of so much milk powder nitrogen in such maintenance rations.

Osborne and Mendel are, therefore, in error in assuming that only the "protein" nitrogen of their protein-free milk is of value, and the facts pointed out above compel us to put a new interpre-

<sup>8</sup> E. V. McCollum and M. Davis: *this Journal*, xx, p. 415, 1915.

<sup>9</sup> McCollum and Davis: *loc. cit.*

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tation on much of their data. They have fed purified protein, not as the sole source of nitrogen, but have superimposed these pure proteins upon nearly half of the maintenance needs of the animals as milk nitrogen. While in some instances this may not detract from the value of these excellent studies, they are certainly not to be considered in the same category with efforts to secure normal nutrition with purified foodstuffs.

Especially untrustworthy is the interpretation which they put upon their experiments in which they claim to have established such fundamental truth as the possibility of maintenance without lysine, by successfully maintaining rats through long periods on rations in which gliadin was superimposed upon protein-free milk containing 1.22 per cent of N x 6.25. We have no reason to suppose that such rations are lysine-free, and the data obtained with such rations in which about 6 per cent of the nitrogen of the food was derived from milk cannot be interpreted as establishing the conclusion drawn from them by these investigators. As far as we are aware, all the curves of their rats fed gliadin without "natural protein-free milk" or rat feces, both of which are the source of a supplementary nitrogen supply, show a downward trend, indicating only a partial maintenance with this protein.

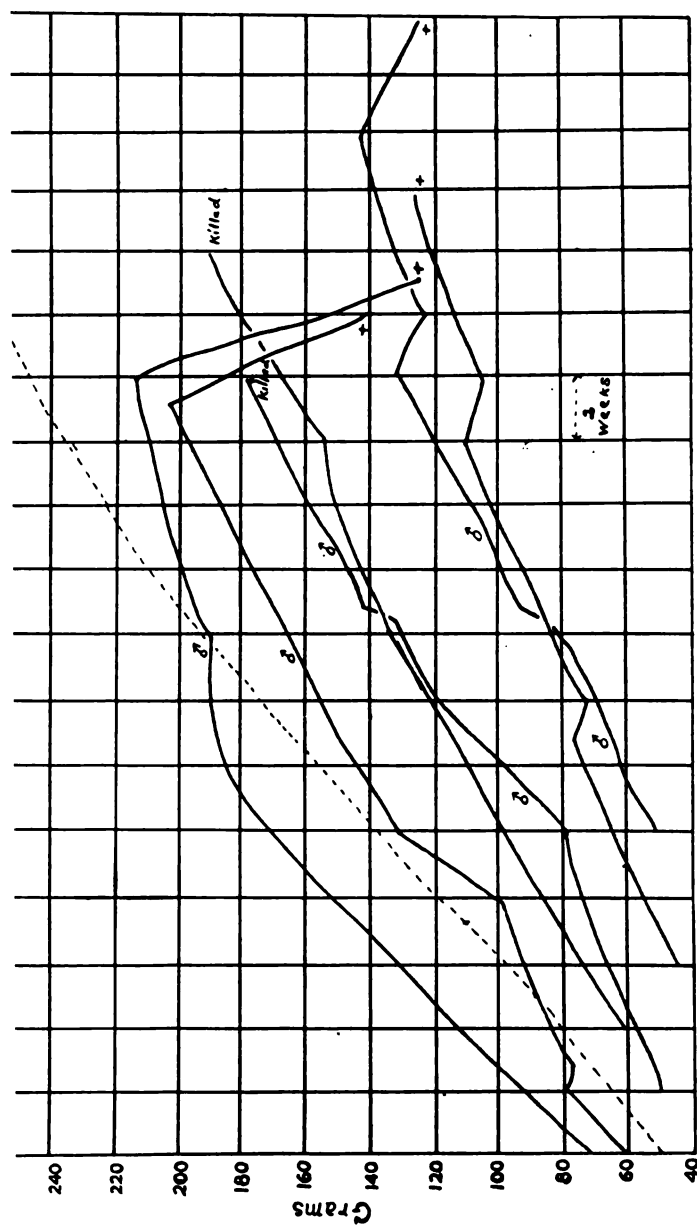


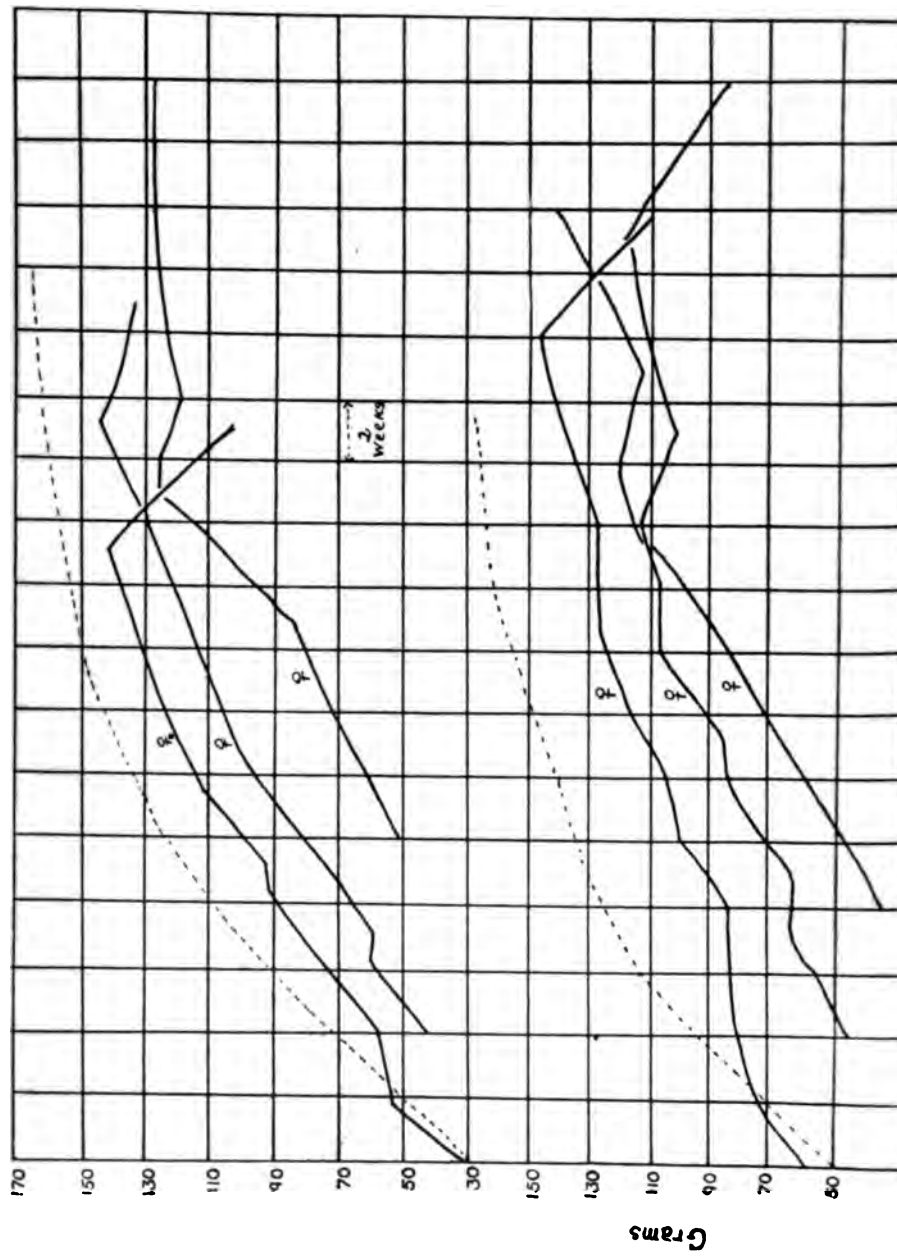
CHART I. Shows the behavior of rats fed a fat-free ration of casein, dextrin, lactose, agar-agar, and a salt mixture. The abrupt failure is characteristic of animals fed such rations. The ration consisted of:

	per cent	The salt mixture employed had the following composition:	gm.
Casein.....	18.0		
Dextrin.....	56.3	NaCl.....	0.173
Lactose.....	20.0	MgSO <sub>4</sub> (anhydrous).....	0.266
Agar-agar.....	2.0	CaH <sub>2</sub> (PO <sub>4</sub> ) + H <sub>2</sub> O.....	1.300
Salt mixture.....	3.7	Fe lactate (Merck).....	0.118
		K <sub>2</sub> HPO <sub>4</sub> .....	0.954

The normal expectation of growth is represented in these charts by a broken line.



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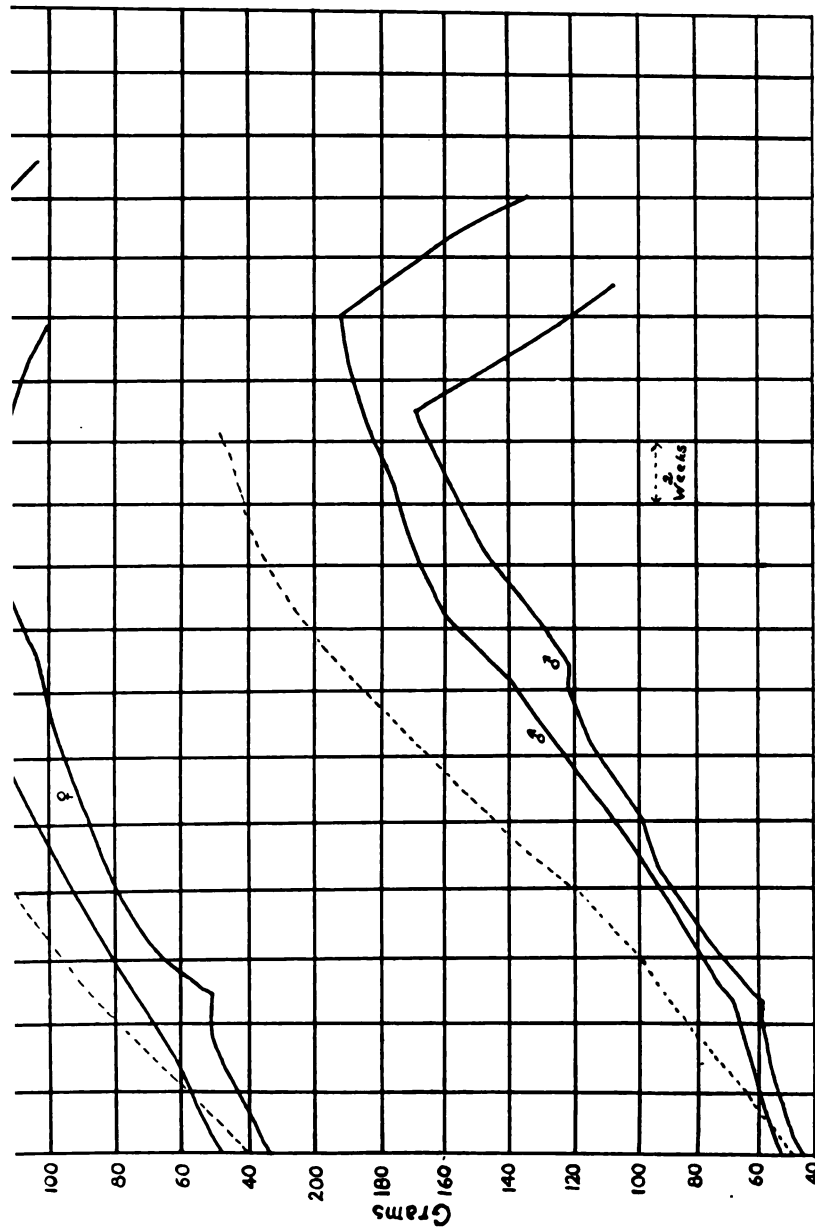


CHART III. Shows the behavior of rats fed the ration described under Chart I, but with 5 per cent cottonseed oil added three times a week. The shape of the curves of growth and decline on this ration is strikingly similar to those of the rats on a fat-free diet (Charts I and II).

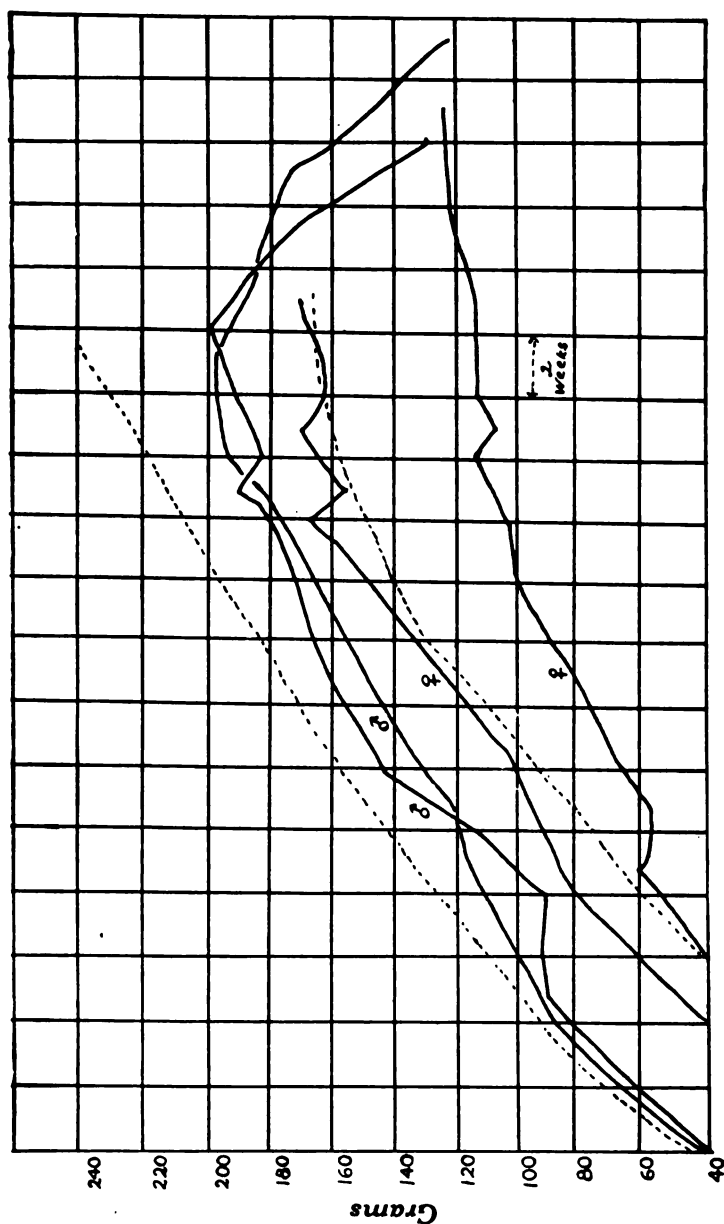


CHART IV. Shows the behavior of rats fed the ration of purified foodstuffs described under Chart I, but with 5 per cent olive oil replacing an equivalent amount of dextrin. The ration given these rats was exactly like that fed in Chart VIII, except that in the latter case butter-fat replaced the olive oil. It is significant that the curves are strikingly similar to those of vigorous rats which have grown on a fat-free diet. The nutritional failure appears after the lapse of about six months.

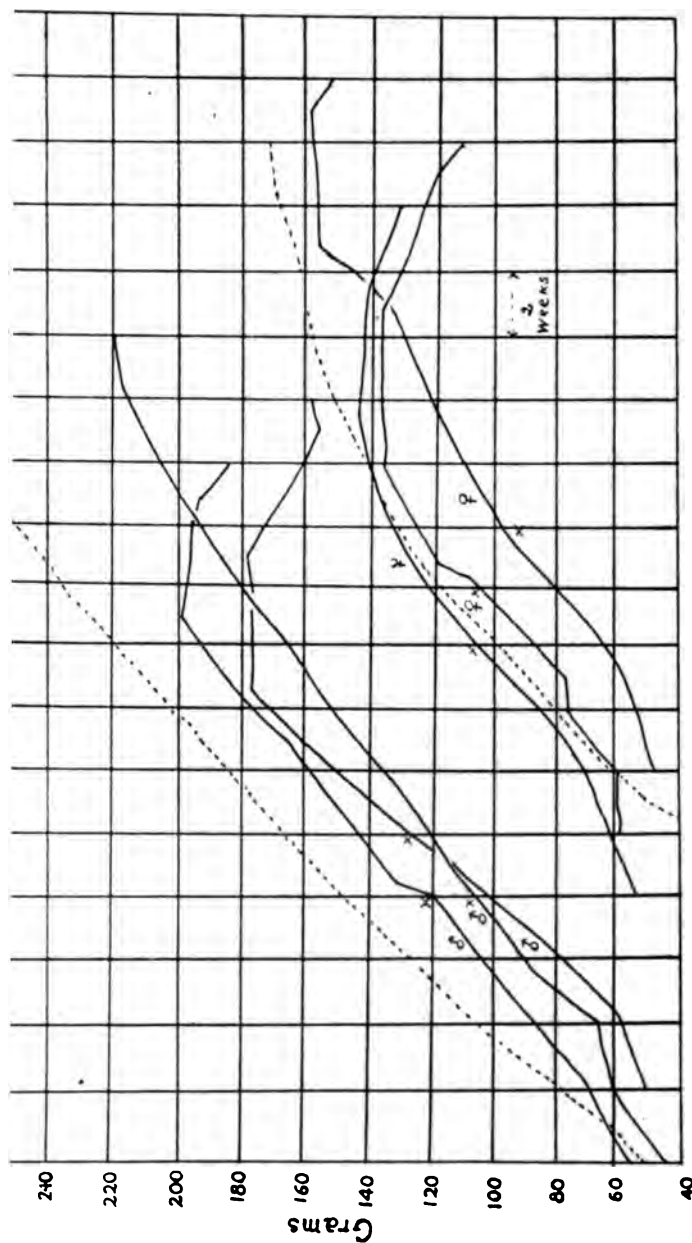
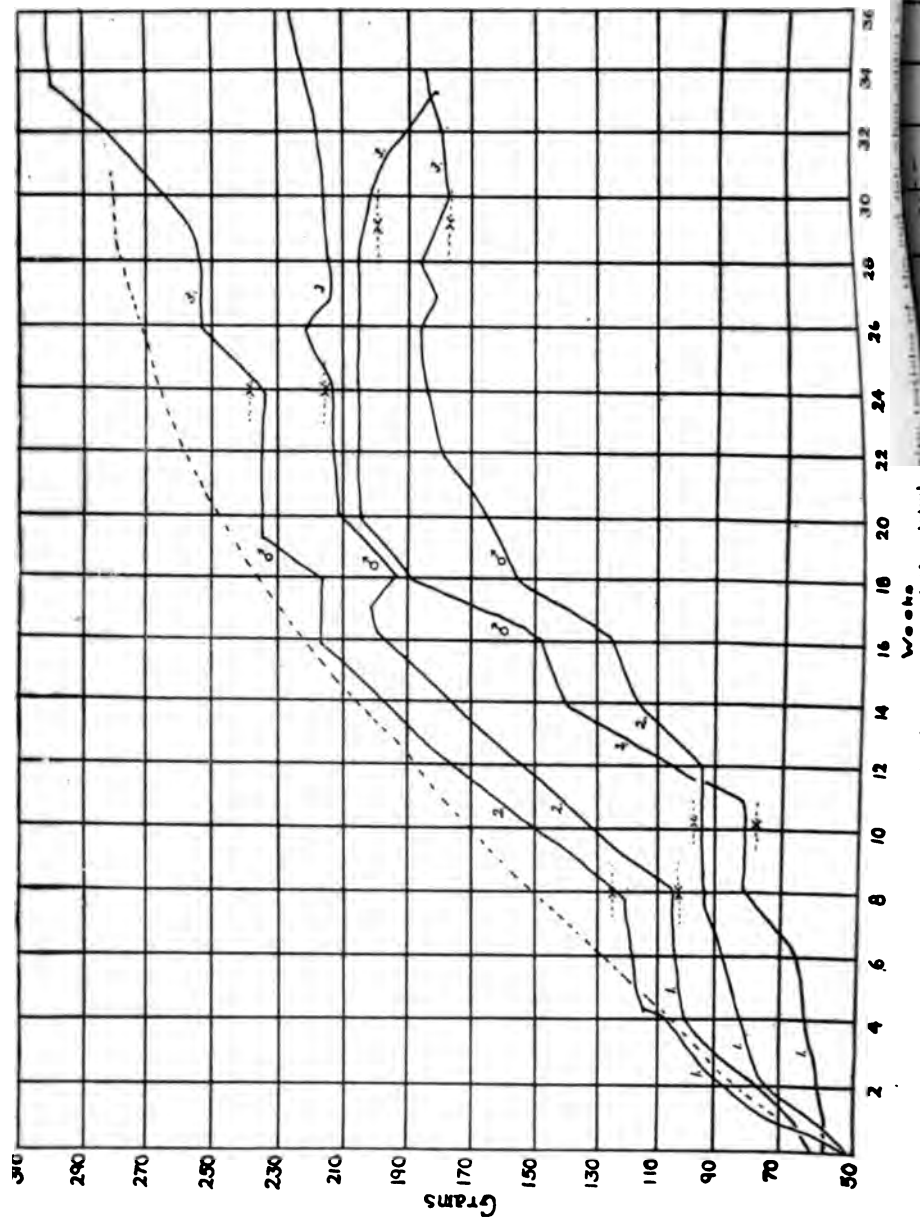


CHART V. Shows the records of rats which received during Period 1 the fat-free diet used throughout the experiments here described. Thereafter the ration contained 1 per cent of butter-fat. It would appear from the behavior of this group of animals that this amount of butter-fat is too low to supply the unknown substance so necessary for long continued growth. However, we would reserve definite conclusions, because it is not known whether the preliminary fat-free period was responsible for the final result. Further experiments are in progress to determine the minimum quantity of butter-fat which is essential for normal nutrition when supplied regularly from the beginning.

A cross marks the termination of Period 1.



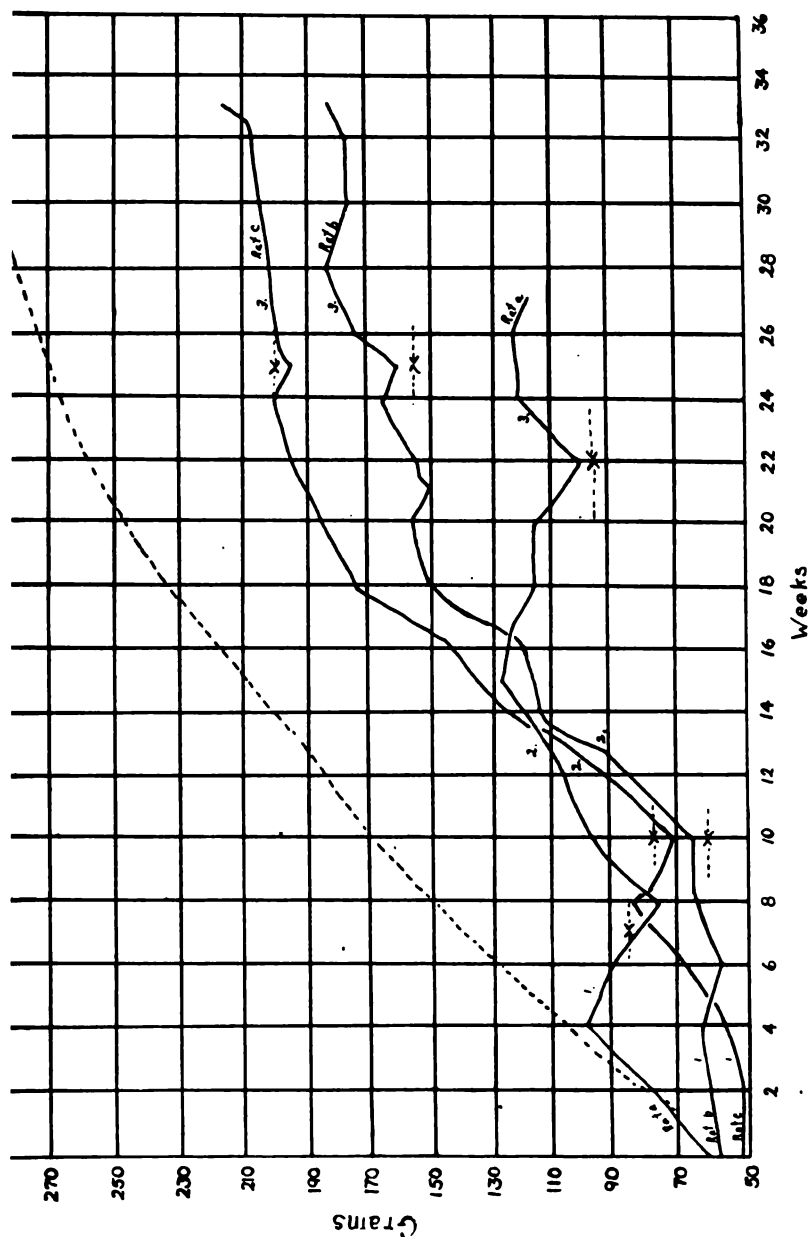
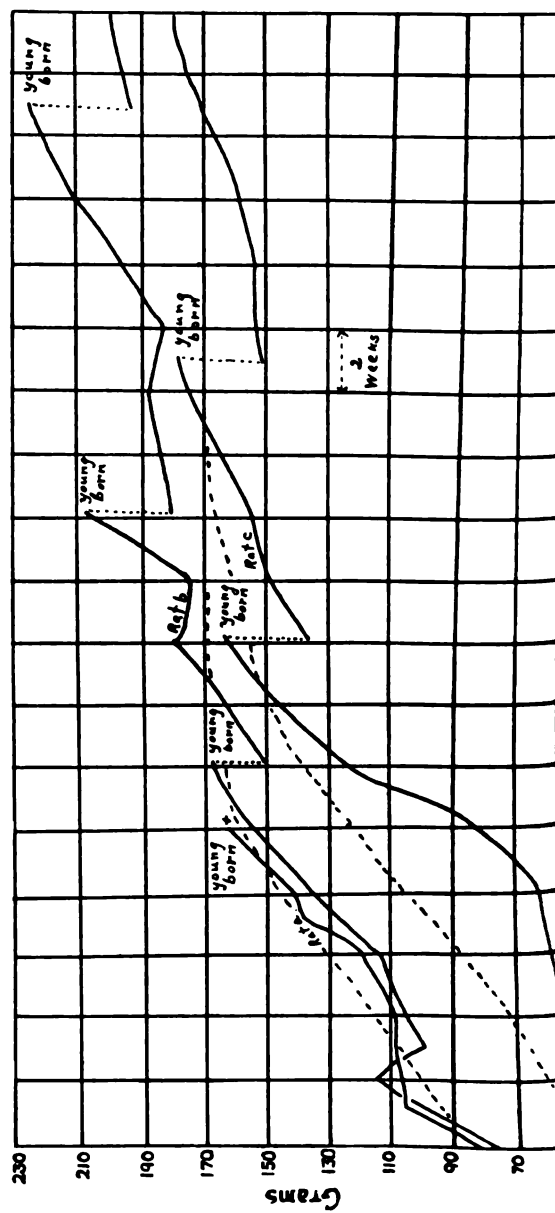


CHART VII. Shows the influence of adding the ether extract of dried pig kidney to the ration employed in Charts I and II. Period 1 was fat-free. In Period 2 the extract of kidney was given. In Period 3 butter-fat (5 per cent) replaced the kidney. There was a marked increase in body weight extending over about 100 days as a result of feeding kidney fat. In two of the three animals there was no noticeable change in their condition as the result of changing from kidney-fat to butter-fat feeding.

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and died immediately thereafter.

Rat B produced two young in the first litter, both of which were normal in size at the age of twenty days. One died on the twentieth day. The other lost weight slightly (dropped from 30 to 27 gm.) during the first eleven days after being placed on the mother's diet, so it was changed to another ration on which it thrived.

Her second litter consisted of eight young. All did well during the suckling period, but they suffered some disturbance in nutrition as a result of changing directly from the mother's milk to the mother's ration of purified foodstuffs. One died at the age of forty-six days. A second died a few days later, and a portion of the head was eaten by its companions. One other was killed at this age because of its poor condition. The other five suffered somewhat from the character of the diet, but ultimately entirely recovered and are now in good condition at the age of 117 days, having never had anything to eat except the ration of purified foodstuffs which had nourished the mother.

The third litter of young produced by Rat B consisted of two young. They are at this writing twenty-one days old and together weigh 54 gm.

Rat C has produced two litters. The first of four young all thrived and have come up successfully on the mother's ration. Their curves of growth are given in Chart IX. The second litter of three all died at twenty-nine days old of malnutrition as a result of being fed the ration of the mother immediately after weaning. Little rats frequently have digestive disturbances when placed upon such diets at a very early age.



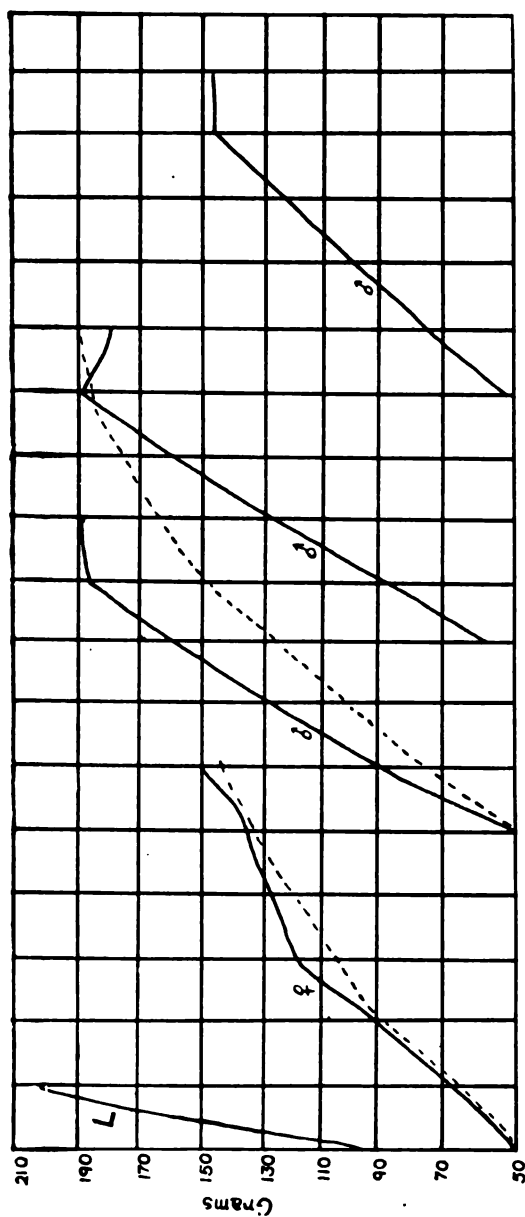


CHART IX. Shows the growth curves of the young of Rat C (Chart VIII). These young were born September 17, 1914. The curve marked L represents the growth of the four young collectively from the sixteenth to the thirtieth day after birth. The other curves illustrate the growth of the individuals from the thirty-fifth day of life.

## THE QUATERNARY SALTS OF HEXAMETHYLENE-TETRAMINE.

### I. SUBSTITUTED BENZYL HALIDES AND THE HEXAMETHYLENE-TETRAMINIUM SALTS DERIVED THEREFROM.

By WALTER A. JACOBS AND MICHAEL HEIDELBERGER.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

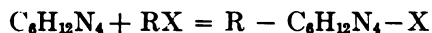
(Received for publication, February 27, 1915.)

Since its introduction into medicine by Nicollai in 1894, hexamethylenetetramine has attracted considerable attention as an internal antiseptic. A number of workers have observed the interesting fact that, in spite of its rapid elimination in the urine after ingestion, it is to be found in most of the body fluids, including even the cerebrospinal fluid. The unusual diffusibility of the drug, combined with its antiseptic properties, has led many to hope for its successful use in infections other than those of the urinary tract. Recent investigations, however, have fairly well established that hexamethylenetetramine in itself is devoid of antiseptic properties and owes its action to the liberation from it of formaldehyde. The prerequisite for this cleavage is that the fluid in which it occurs should be acid or perhaps contain a suitable enzyme. Besides, granting that cleavage occurs, enough of the drug must gain access to the locality desired in order to yield formaldehyde in a concentration sufficient to be effective. Owing, among other things, to the alkalinity of most of the body fluids, it would seem that there are serious limitations to the use of the drug other than as a urinary antiseptic.

In the present investigations, which were inspired by the interest of Dr. Simon Flexner in hexamethylenetetramine as a possible therapeutic agent in the treatment of poliomyelitis, it seemed to the authors that these limitations might be overcome by suitable chemical variation. For other reasons many investigators have already attempted improvements in the action of hexamethylenetetramine by combining it in the form of complex

salts with acids, metallic salts, phenols, etc., but such compounds were discarded by the authors on the ground that the molecule of the base itself was not altered. This is evident from its easy regeneration from such compounds, solution in water often sufficing to produce hydrolysis. At the start of the present work it was found that by a more fundamental alteration of the hexamethylenetetramine molecule by the introduction of chemical groups in the manner described below, this drug could be converted into substances with a definite bactericidal power. This power, as regards magnitude and specificity, was determined by the character of the entering group. A detailed discussion of the biological results of the work we shall defer to a later communication, but for the present suffice it to say that these substances represent a new group of organic bactericides.

As is well known, hexamethylenetetramine functions as a tertiary base and as such reacts with certain types of organic halides to form quaternary salts, according to the equation



only one molecule of halide reacting with one of the base. This property forms the basis of the work to be described. A search of the literature revealed that but comparatively little had been done in this direction, although enough to indicate the chemical possibilities. Wohl<sup>1</sup> was the first to prepare the methyl and ethyl salts, proving thereby the tertiary character of the base. Delépine<sup>2</sup> later employed the allyl, amyl, and benzyl salts as the basis of a very useful method for the preparation of the corresponding primary amines. Mannich and his collaborators<sup>3</sup> succeeded in applying this method to the synthesis of several aminoketones and glycinephenolic esters, while Einhorn and Göttler<sup>4</sup> prepared the hexamethylenetetraminium salts of the halogenacetamides and their oxymethyl derivatives. Sommelet<sup>5</sup> has also used hexamethylenetetraminium compounds in an interesting synthesis of

<sup>1</sup> Wohl: *Ber. d. deutsch. chem. Gesellsch.*, xix, pp. 1843-44, 1886.

<sup>2</sup> Delépine: *Bull. Soc. chim.*, series 3, xiii, p. 356, 1895; xvii, p. 293, 1897.

<sup>3</sup> Mannich and Hahn: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 1542, 1911.  
Mannich and Drauzberg: *Arch. Pharm.*, ccl, p. 532, 1912.

<sup>4</sup> Einhorn and Göttler: *Ann. d. Chem.*, cclxi, p. 150, 1908.

<sup>5</sup> Sommelet: *Compt. rend. Acad. d. sc.*, clvii, 852, 1913.

aldehydes and ketones. The description of the salts of the base with the halogenacetic esters,<sup>6</sup> bromo- and iodo-ethylphthalimide and bromoethylcarbonylsalicylamide,<sup>7</sup> and chloroacetyl-*p*-phenetidine<sup>8</sup> completes the list, as far as the writers are aware.

In the work to be presented it will be shown that the reaction between hexamethylenetetramine and substances containing aliphatically bound halogen resulting in the formation of quaternary salts is capable of very wide extension. By this means it has been found possible to assemble for chemotherapeutic study the most widely varying types of organic radicals in a comparable, water-soluble form.

The chemical work often necessitated the preparation of new intermediate products required for the synthesis of the final quaternary salts, and where this has occurred the descriptions will be incorporated in the experimental part along with the final product. The number of preparations is so large that it has been deemed advisable for the sake of clearness and accessibility to divide the material as consistently as possible according to chemical type and to make it the subject of several papers. In the following there will be described a number of new benzyl alcohols and chlorides together with the hexamethylenetetramine derivatives obtained therefrom and from already known benzyl halides.

#### EXPERIMENTAL.

To avoid unnecessary repetition, it is to be understood that unless otherwise stated the hexamethylenetetraminium salts described in this and the following papers were made in the following manner: Equimolecular amounts of halide and hexamethylenetetramine were boiled in dry chloroform (5 to 7 cc. for each gram of base), until after solution of the components the amount of product which separated had reached a maximum. This occurred usually in one-half to two hours. In some cases addition occurred so readily that the mixture warmed spontaneously. The product was then filtered off, and upon boiling the filtrate more was often obtained. Occasionally the reaction product

<sup>6</sup> Locquin: *Bull. Soc. chim.*, series 3, xxiii, p. 661, 1900.

<sup>7</sup> D. R. P. 164510; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, viii, p. 921, 1905-7.

<sup>8</sup> D. R. P. 264263; *Chem. Centralbl.*, lxxxiv, pt. ii, p. 1179, 1913.

formed supersaturated solutions which required manipulation to induce crystallization. The product was washed with dry chloroform followed by dry acetone and dried in a desiccator over sulphuric acid or calcium chloride and paraffin. If care was taken to start with absolutely pure material the products so obtained were also pure. Otherwise, except in a few cases, purification by recrystallization was impossible. When this was attempted from alcohol or water, decomposition almost invariably occurred, and the general insolubility of the products in water-immiscible solvents naturally limited further attempts. The yields were good unless otherwise stated.

Halogen determinations were made by the Volhard method, except in the case of compounds giving highly colored solutions or giving colors with the indicator used. For iodides the method was modified by the addition first of an excess of standard silver nitrate solution before adding the indicator and then titrating back the excess. In the case of the intermediate halides described in this and the following papers it was usually found sufficient to boil the substance for several hours with alcoholic sodium hydroxide and then to determine the halogen by the Volhard method. In most cases the melting points of the salts varied greatly with the rapidity of heating, so that the uniform procedure was adopted of heating rapidly to within a few degrees of the melting point and then more slowly. For this reason any attempt at corrected melting points was futile.

In many cases deviations from the above procedures were found necessary and these will of course be denoted below as they occur.

Before proceeding to a description of the individual substances, a characteristic property of the salts containing aromatic radicals may be mentioned. When the aqueous solutions are allowed to stand or are warmed, formaldehyde is liberated and a product separates as a colloidal suspension, an emulsion of oil drops, or a crystalline precipitate, depending upon the salt used. These precipitates are usually soluble in acids, and where isolation was possible, have been shown to consist principally of the methylene compounds of the corresponding primary amines. A description of these substances and of their biological properties will be the subject of a later communication.

*o*-Methylbenzylhexamethylenetetraminium chloride. Diamond-shaped micro-plates, melting at 202–4° with preliminary darkening and softening. Easily soluble in water and alcohol.

0.1997 gm. of substance required 7.05 cc. AgNO<sub>3</sub> Solution I.\*

Calculated for C<sub>14</sub>H<sub>21</sub>N<sub>4</sub>Cl: Cl = 12.63 per cent.

Found: Cl = 12.42 per cent.

*m*-Methylbenzylhexamethylenetetraminium chloride. Dry acetone was added to the solution containing the partially precipitated salt in order to complete the separation. After boiling with dry acetone the product melted at 204–5° with decomposition and preliminary darkening, and dissolved readily in water.

0.2036 gm. of substance required 7.27 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>14</sub>H<sub>21</sub>N<sub>4</sub>Cl: Cl = 12.63 per cent.

Found: Cl = 12.57 per cent.

*p*-Methylbenzylhexamethylenetetraminium chloride. The product obtained by the interaction of equimolecular amounts of *p*-methylbenzyl chloride and hexamethylenetetramine in boiling chloroform separated incompletely as an oil. In order to obtain the substance crystalline, the chloroform was boiled off and the residue treated with dry acetone. The product was pulverized and again boiled with dry acetone. It sinters and turns yellow at 186°, melts at 198°, and is soluble in alcohol, chloroform, and very readily in water.

0.2036 gm. of substance required 7.20 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>14</sub>H<sub>21</sub>N<sub>4</sub>Cl: Cl = 12.63 per cent.

Found: Cl = 12.45 per cent.

3, 5-Dimethylbenzylhexamethylenetetraminium chloride. The addition of several volumes of dry acetone was necessary to complete the precipitation of the salt. It melts at 203–4° with decomposition, and is easily soluble in water, alcohol, and chloroform.

0.1480 gm. of substance required 5.19 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>14</sub>H<sub>21</sub>N<sub>4</sub>Cl: Cl = 12.03 per cent.

Found: Cl = 12.34 per cent.

*o*-Xylylenedi-hexamethylenetetraminium dichloride. A solution of 1 gram of *o*-xylylene chloride in dry chloroform was added to

\* 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

a solution of 1.8 grams of hexamethylenetetramine in the same solvent. After twenty-four hours the crystals of the salt were filtered off, boiled with dry acetone, and dried *in vacuo* at room temperature. The substance turns gray on heating and decomposes at 162°. It is very soluble in water, the concentrated aqueous solution turning dark brown on boiling and depositing brown flocks.

0.1337 gm. of substance required 11.04 cc. AgNO<sub>3</sub> Solution II.<sup>10</sup>

Calculated for C<sub>20</sub>H<sub>32</sub>N<sub>4</sub>Cl<sub>2</sub>: Cl = 15.57 per cent.

Found: Cl = 15.36 per cent.

Attempts to hasten the preparation of the salt by heating resulted in decomposition.

*m-Xylylenedi-hexamethylenetetraminium dichloride.* The salt was prepared in the same way as the preceding compound. It melts at 180-6° and is very soluble in water.

0.2103 gm. of substance required 9.04 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>20</sub>H<sub>32</sub>N<sub>4</sub>Cl<sub>2</sub>: Cl = 15.57 per cent.

Found: Cl = 15.13 per cent.

*Mesityldi-hexamethylenetetraminium dichloride.* The salt is easily soluble in water and melts with decomposition at 188-91°.

0.1977 gm. of substance required 8.48 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>21</sub>H<sub>24</sub>N<sub>4</sub>Cl<sub>2</sub>: Cl = 15.11 per cent.

Found: Cl = 15.10 per cent.

*β-Naphthobenzylhexamethylenetetraminium chloride.* A crude β-chloromethylnaphthalene was used, resulting in a poor yield. The crude product was again boiled out with chloroform in order to remove unchanged hexamethylenetetramine. Attempts to recover more of the salt from the mother liquors resulted only in the isolation of hexamethylenetetraminium chloride. The new salt melts at 190-5° after turning yellow, and is easily soluble in water and soluble in alcohol.

0.2057 gm. of substance required 6.85 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>17</sub>H<sub>21</sub>N<sub>4</sub>Cl: Cl = 11.20 per cent.

Found: Cl = 11.72 per cent.

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<sup>10</sup> 1 cc. = 0.00186 gm. Cl; 0.004192 gm. Br; 0.00666 gm. I.

*o*-Chlorobenzylhexamethylenetetraminium chloride. Minute, glistening plates, easily soluble in water and melting at 201–3° with decomposition.

0.2255 gm. of substance required 7.40 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>13</sub>H<sub>18</sub>N<sub>4</sub>Cl<sub>2</sub>: Cl<sup>-</sup> = 11.77 per cent.

Found: Cl<sup>-</sup> = 11.55 per cent.

*p*-Chlorobenzylhexamethylenetetraminium chloride. Micro-prisms, very soluble in water and melting at 202–5° with decomposition.

0.2063 gm. of substance required 6.76 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>13</sub>H<sub>18</sub>N<sub>4</sub>Cl<sub>2</sub>: Cl<sup>-</sup> = 11.77 per cent.

Found: Cl<sup>-</sup> = 11.54 per cent.

*o*-Bromobenzyl chloride. The theoretical amount of chlorine was passed into boiling *o*-bromotoluene and the product fractionated *in vacuo*. The chloride boils at 124–6° (corrected) at 20 mm.

0.2064 gm. of substance required 19.0 cc. AgNO<sub>3</sub> Solution II.

Calculated for C<sub>7</sub>H<sub>5</sub>ClBr: Cl = 17.25 per cent.

Found: Cl = 17.12 per cent.

*o*-Bromobenzylhexamethylenetetraminium chloride. Micro-crystals, soluble in water and melting at 183–6° with decomposition.

0.2064 gm. of substance required 5.77 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>13</sub>H<sub>18</sub>N<sub>4</sub>ClBr: Cl = 10.26 per cent.

Found: Cl = 9.84 per cent.

*p*-Bromobenzylhexamethylenetetraminium chloride. Persistently low results were obtained on analysis of this substance, possibly owing to the stubborn retention of solvents, as the various preparations all melted at the same point. The best result was that given below, obtained after recrystallizing from absolute alcohol. Micro-crystals, rather difficultly soluble in water and melting at 192° with decomposition.

0.2385 gm. of substance required 6.54 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>13</sub>H<sub>18</sub>N<sub>4</sub>ClBr: Cl = 10.26 per cent.

Found: Cl = 9.65 per cent.

*p*-Iodobenzylhexamethylenetetraminium bromide. The salt separates in the cold on mixing chloroform solutions of the components and melts with decomposition at 181–2°. It is very difficultly soluble in water.



0.3341 gm. of substance (Kjeldahl) required 31.0 cc.  $\frac{N}{16}$  HCl.

0.4023 gm. of substance gave 0.2191 gm. AgBr.

Calculated for  $C_{11}H_{18}N_4BrI$ : N = 12.82 per cent; Br = 19.29 per cent.

Found: N = 12.99 per cent; Br = 18.49 per cent.

*o*-Cyanobenzylhexamethylenetetraminium chloride. Thick, irregular micro-plates, very soluble in water and melting at  $183^\circ$  with decomposition and preliminary darkening.

0.2306 gm. of substance required 8.08 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{11}H_{18}N_4Cl$ : Cl = 12.15 per cent.

Found: Cl = 12.02 per cent.

*p*-Cyanobenzylhexamethylenetetraminium chloride. It was necessary to boil the substance one hour with dry acetone in order to remove chloroform which was retained even on drying to constant weight *in vacuo* at  $100^\circ$  over sulphuric acid. Glistening micro-plates, much more difficultly soluble in water than the *ortho* isomer, and melting at  $193-3.5^\circ$  with decomposition.

0.2020 gm. of substance required 7.00 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{11}H_{18}N_4Cl$ : Cl = 12.15 per cent.

Found: Cl = 12.20 per cent.

*o*-Nitrobenzylhexamethylenetetraminium chloride. Crystals, turning orange at  $175-6^\circ$  and decomposing at  $178-9^\circ$ ; easily soluble in water.

0.2105 gm. of substance required 6.75 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{11}H_{18}O_2N_4Cl$ : Cl = 11.38 per cent.

Found: Cl = 11.29 per cent.

*m*-Nitrobenzylhexamethylenetetraminium chloride. Long continued boiling of the chloroform solution of the components was necessary in order to obtain a good yield. The salt forms glistening micro-prisms, melting at  $199-202^\circ$  with decomposition, and readily soluble in water.

0.2159 gm. of substance required 6.69 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{11}H_{18}O_2N_4Cl$ : Cl = 11.38 per cent.

Found: Cl = 10.91 per cent.

*p*-Nitrobenzylhexamethylenetetraminium chloride. Crystals, melting, and decomposing at  $173-4^\circ$ . Readily soluble in water.

0.2388 gm. of substance gave 0.1120 gm. AgCl.  
 Calculated for  $C_{12}H_{14}O_2N_2Cl$ : Cl = 11.38 per cent.  
 Found: Cl = 11.60 per cent.

*p*-Nitrobenzylpyridinium chloride. This salt, already recorded by Lellmann and Pekrun,<sup>11</sup> was prepared for purposes of comparison. These authors describe the compound as yellow and give 103° as the melting point, whereas our preparation formed almost colorless, diamond-shaped prisms, softening at 203° and melting at 204–7° to a red liquid.

0.2417 gm. of substance required 9.65 cc. AgNO<sub>3</sub> Solution I.  
 Calculated for  $C_{12}H_{11}O_2N_2Cl$ : Cl = 14.15 per cent.  
 Found: Cl = 14.05 per cent.

2, 4-Dinitrobenzylhexamethylenetetraminium chloride. Faintly yellow micro-platelets, melting and decomposing at 178–9° and difficultly soluble in water.

0.2089 gm. of substance required 10.83 cc. AgNO<sub>3</sub> Solution II.  
 Calculated for  $C_{12}H_{17}O_4N_6Cl$ : Cl = 9.94 per cent.  
 Found: Cl = 9.65 per cent.

*o*-Acetaminobenzyl chloride. Lellmann and Pekrun's<sup>12</sup> method for the preparation of *o*-aminobenzylpiperidine was modified as follows: 39.7 grams of *o*-nitrobenzyl chloride were dissolved in absolute alcohol and poured into a solution of 42 grams of piperidine in two volumes of absolute alcohol. After boiling the solution about three hours, most of the alcohol was removed *in vacuo*, the residue diluted with water, and the precipitated oil extracted with ether. The ethereal solution of crude *o*-nitrobenzylpiperidine was then cautiously treated with concentrated hydrochloric acid in excess, finally with shaking, and the lower layer gradually run into a solution of 180 grams of stannous chloride in 450 cc. of concentrated hydrochloric acid. After warming on the water bath for one-half hour and letting stand over night, the base was separated by means of sodium hydroxide and extracted with ether. Contrary to the experience of Lellmann and Pekrun, the base was not readily volatile with steam and did not crystallize in the receiver. The ether was therefore simply removed and the crude

<sup>11</sup> Lellmann and Pekrun: *Ann. d. Chem.*, cclix, p. 57, 1890.

<sup>12</sup> Lellmann and Pekrun: *loc. cit.*, p. 46.

residue acetylated according to Kühn.<sup>12</sup> The crude *o*-acetaminobenzylpiperidine separated as an oil which soon solidified, and could be recrystallized by dissolving in hot alcohol and adding water to incipient turbidity. Yield: 45 grams, melting at 76–7°. This was converted into *o*-acetaminobenzyl chloride by Kühn's method, it being found more advantageous, however, to isolate the product by evaporating the chloroform-toluene solution to about one-quarter volume and removing the carbopiperidide by shaking out with dilute hydrochloric acid, whereupon the *o*-acetaminobenzyl chloride crystallized out spontaneously.

*o*-Acetaminobenzylhexamethylenetetraminium chloride. Micro-platelets, easily soluble in water and melting at 180–2° with decomposition.

0.3579 gm. of substance gave 0.1561 gm. AgCl.

Calculated for  $C_{11}H_{12}ON_4Cl$ : Cl = 10.96 per cent.

Found: Cl = 10.79 per cent.

*p*-Acetaminobenzyl chloride. This was prepared in essentially the same way as the *ortho* isomer, except that the solution of *p*-nitrobenzyl chloride and piperidine was heated for only one hour, nor was it necessary to heat it in order to reduce the *p*-nitrobenzylpiperidine completely. The *p*-acetaminobenzyl chloride could be obtained in crystalline form only after complete removal of the chloroform-toluene mixture *in vacuo* and taking up in hot benzene.

*p*-Acetaminobenzylhexamethylenetetraminium chloride. Several hours' boiling was necessary in this case to obtain an almost quantitative yield. Micro-prisms, melting at 175–7° with decomposition, and readily soluble in water.

0.2433 gm. of substance gave 0.1070 gm. AgCl.

Calculated for  $C_{11}H_{12}ON_4Cl$ : Cl = 10.96 per cent.

Found: Cl = 10.88 per cent.

An attempt was made to prepare 3-acetamino-4-acetoxybenzyl chloride, but was abandoned during the later stages of the synthesis owing to the poor yields obtained and pressure of other work. The following three compounds were encountered during this attempt.

<sup>12</sup> Kühn: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2900, 1900.

**3-Nitro-4-oxybenzylpiperidine.** The method of Auwers and Dombrowski<sup>14</sup> for the preparation of this substance is slow and gives a poor yield. An alcoholic solution of 50 grams of 3-nitro-4-oxybenzyl chloride and 50 grams of piperidine was boiled for four hours. The solution was then concentrated *in vacuo* until an abundant precipitation of yellow needles occurred. Examination of this substance showed that it was a hydrochloride of complex nature. On standing over night in contact with the solution, hydrolysis occurred, and the liquid was filled with the characteristic orange-brown needles (not red, as stated by Auwers and Dombrowski) of 3-nitro-4-oxybenzylpiperidine. More was obtained by saturating the concentrated mother liquors with carbon dioxide, recrystallization from alcohol being necessary to remove the accompanying salts. Total yield: 45.2 grams, melting at 141–3° with preliminary softening.

0.1947 gm. of substance gave 20.25 cc. N (764 mm. and 14.5°).

Calculated for  $C_{12}H_{14}O_2N_2$ : N = 11.87 per cent.

Found: N = 12.22 per cent.

**3-Nitro-4-acetoxybenzylpiperidine hydrochloride.** The above compound was acetylated by treatment with acetyl chloride in pyridine solution. After standing for several hours, the hydrochloride of the ester was precipitated with dry ether and reprecipitated from dry acetone with dry ether. The substance separated as faintly yellow, glistening micro-platelets, melting at 178–9°, very soluble in water and alcohol, and easily hydrolyzed to the phenol.

0.2010 gm. of substance required 6.29 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{14}H_{16}O_4N_2 \cdot HCl$ : Cl = 11.27 per cent.

Found: Cl = 11.02 per cent.

**3-Amino-4-oxybenzylpiperidine dihydrochloride.** The nitro compound was reduced with stannous chloride in concentrated hydrochloric acid. After warming for one-half hour on the water bath, the mixture was cooled with ice and the double tin salt filtered off. This was decomposed with hydrogen sulphide and the hydrochloride isolated from the filtrate in the usual manner. The hot alcoholic extract of the crude salt was bone-blackened and precipi-

<sup>14</sup> Auwers and Dombrowski: *Ann. d. Chem.*, cccxlv, p. 289, 1906.

tated with ether. Yield: about 40 per cent of the weight of starting material. When recrystallized as above, the salt formed microscopic, hexagonal plates, softening above 135° and evolving gas at about 180°. The aqueous solution gives a deep red color with ferric chloride, changing to orange-brown. The low analytical result is possibly explained by loss of hydrochloric acid during drying *in vacuo*.

0.2174 gm. of substance gave 0.2125 gm. AgCl.

Calculated for  $C_{11}H_{11}ON_2 \cdot 2HCl$ : Cl = 25.41 per cent.

Found: Cl = 24.18 per cent.

*o*-Acetoxymesityl pseudochloride (*2*-acetoxy-3, 5-dimethylbenzyl chloride). *o*-Oxymesityl pseudochloride<sup>15</sup> was dissolved in an excess of acetic anhydride, treated with a few drops of sulphuric acid, and heated for fifteen minutes on the water bath. After cooling and shaking with water, the resulting oil was shaken out with ether, dried, evaporated to small bulk, and distilled *in vacuo*. The fraction boiling from 160–70° at 12 mm. crystallized on freezing, and after two recrystallizations from ligroin with the aid of a freezing mixture, melted at 37.5–38° (corrected), with slight preliminary softening.

0.2911 gm. of substance required 13.65 cc. AgNO<sub>3</sub> Solution I.

Calculated for  $C_{11}H_{11}O_2Cl$ : Cl = 16.68 per cent.

Found: Cl = 16.51 per cent.

*2*-Acetoxy-3,5-dimethylbenzylhexamethylenetetraminium chloride. As the substance was soluble in dry chloroform, the solvent was evaporated off and the residue taken up in dry acetone, soon setting to a solid cake. The salt melts at 158° and is easily soluble in water, alcohol, and chloroform.

0.1791 gm. of substance required 5.05 cc. AgNO<sub>3</sub> Solution I.

Calculated for  $C_{17}H_{23}O_2N_4Cl$ : Cl = 10.06 per cent.

Found: Cl = 9.93 per cent.

*2*-Oxy-3,5-dibromobenzylhexamethylenetetraminium bromide. The gummy mass first formed on adding the pseudobromide<sup>16</sup> to a suspension of hexamethylenetetramine in chloroform gradually

<sup>15</sup> Fries and Kann: *ibid.*, cccliii, p. 350, 1907.

<sup>16</sup> Auwers and Schröter: *ibid.*, ccxlv, p. 142, 1906.

became crystalline, after which the product was pulverized and the heating continued. The salt turns yellow above 165°, melts at 173–4°, and is difficultly soluble in water.

0.1978 gm. of substance required 7.75 cc. AgNO<sub>3</sub> Solution II.

Calculated for C<sub>13</sub>H<sub>17</sub>ON<sub>4</sub>Br<sub>2</sub>: Br<sup>-</sup> = 16.48 per cent.

Found: Br<sup>-</sup> = 16.43 per cent.

*2-Acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide.* Crystallization of the salt obtained from the pseudobromide<sup>17</sup> was facilitated by the addition of absolute alcohol. The salt gradually softens above 135°, decomposes at 151–3°, and is difficultly soluble in water.

0.2735 gm. of substance required 5.05 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>13</sub>H<sub>19</sub>O<sub>2</sub>N<sub>4</sub>Br<sub>2</sub>: Br<sup>-</sup> = 15.16 per cent.

Found: Br<sup>-</sup> = 14.66 per cent.

*4-Acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide.* The salt obtained from the pseudobromide<sup>18</sup> formed crystals sintering at 180° and melting at 184–5°, and was difficultly soluble in water.

0.1982 gm. of substance required 3.7 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>13</sub>H<sub>19</sub>O<sub>2</sub>N<sub>4</sub>Br<sub>2</sub>: Br<sup>-</sup> = 15.16 per cent.

Found: Br<sup>-</sup> = 14.81 per cent.

*2-Acetoxy-3, 5-dimethyl-4, 6-dibromobenzylhexamethylenetetraminium bromide.* Dibromo-*o*-acetoxymesityl bromide<sup>19</sup> and hexamethylenetetramine reacted vigorously in dry, boiling chloroform, but, as no product separated, the salt was precipitated with dry ether and purified by boiling with dry acetone. It melts at 150–1° and is difficultly soluble in water.

0.2034 gm. of substance required 3.7 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>17</sub>H<sub>23</sub>O<sub>2</sub>N<sub>4</sub>Br<sub>2</sub>: Br<sup>-</sup> = 14.40 per cent.

Found: Br<sup>-</sup> = 14.43 per cent.

*2-Oxy-5-nitrobenzylhexamethylenetetraminium chloride.* The salt was prepared from 2-oxy-5-nitrobenzyl chloride<sup>20</sup> and hexa-

<sup>17</sup> Auwers and Büttner: *ibid.*, cccii, p. 150, 1898.

<sup>18</sup> Auwers and Daেকে: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 3377, 1899.

<sup>19</sup> Fries and Kann: *Ann. d. chem.*, cccliii, p. 342, 1907.

<sup>20</sup> D. R. P. 132475. *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, vi, p. 142, 1900–02.

## 672 Salts of Hexamethylenetetramine

methylenetetramine in dry chloroform in a sealed tube for one-half hour at 100° and purified by boiling with dry acetone. Bright yellow powder, melting and decomposing at 210°, and difficultly soluble in water, the solution giving a faint purple-brown color with ferric chloride.

0.3022 gm. of substance gave 0.1270 gm. AgCl.

Calculated for  $C_{11}H_{14}O_2N_4Cl$ : Cl = 10.82 per cent.

Found: Cl = 10.40 per cent.

*3-Nitro-4-acetoxybenzyl chloride.* 10 grams of 3-nitro-4-oxybenzyl chloride<sup>20</sup> were treated with 15 cc. of acetic anhydride and a few drops of sulphuric acid and heated for fifteen minutes on the water bath. After cooling, the mixture was treated with water. An oil separated, which crystallized on standing. Yield: 11 grams after recrystallization from ligroin. For analysis the substance was recrystallized twice from 85 per cent alcohol, forming very faintly yellow, glistening leaflets, melting at 59.5–60° (corrected), and very soluble in benzene and ether.

0.2111 gm. of substance (Kjeldahl) required 9.2 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_9H_8O_4NCl$ : N = 6.10 per cent.

Found: N = 6.10 per cent.

An attempt to prepare the hexamethylenetetraminium salt of this chloride resulted in the formation of a yellow substance which did not give analytical figures agreeing with those calculated. For this reason the chloride was converted into the iodide.

*3-Nitro-4-acetoxybenzyl iodide.* 6 grams of chloride were dissolved in a little dry acetone and treated with a normal solution of sodium iodide in dry acetone<sup>21</sup> (4 grams of NaI). After standing for several days the mixture was poured into water, precipitating an oil which soon solidified. The product was ground up with water, filtered off, dried, and recrystallized by dissolving in a small volume of chloroform and precipitating with petroleum ether (boiling at 40–60°). Yield: 7.6 grams. For analysis the iodide was recrystallized twice from 85 per cent alcohol, forming slender, almost colorless needles, melting at 65.5–68° (corrected), soluble in ether, and very soluble in benzene and chloroform.

<sup>21</sup> Finkelstein: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 1528. 1910.

0.1582 gm. of substance gave 0.1142 gm. AgI; 0.1606 gm. gave 0.1155 gm. AgI.

Calculated for  $C_9H_8O_4NI$ : I = 39.54 per cent.

Found: I = 38.87; 39.00 per cent.

*3-Nitro-4-acetoxybenzylhexamethylenetetraminium iodide.* Chloroform solutions of the components reacted in the cold, the salt separating as an oil which gradually solidified. After warming for a few minutes, the product was filtered off and ground up and washed thoroughly with chloroform. The somewhat impure compound could not be recrystallized, owing to its insolubility. It softens at  $159^\circ$  to a semifluid, orange-colored mass, and is very difficultly soluble in water.

0.2158 gm. of substance gave 0.1144 gm. AgI.

Calculated for  $C_{15}H_{20}O_4N_6I$ : I = 27.52 per cent.

Found: I = 28.66 per cent.

*3-Nitro-6-acetoxybenzyl chloride.* Prepared similarly to its isomer and recrystallized from 95 per cent alcohol, forming minute, colorless platelets, softening above  $55^\circ$  and melting gradually to a liquid which is clear at  $83^\circ$  (corrected), the drag being due to the presence of alcohol of crystallization, as was shown by the iodoform test.

0.1981 gm. of substance gave 9.40 cc. N (761 mm. and  $23^\circ$ ).

Calculated for  $C_9H_8O_4NCl \cdot C_2H_5OH$ : N = 5.08 per cent.

Found: N = 5.33 per cent.

An attempt to prepare the hexamethylenetetraminium salt resulted in this case, also, in the formation of a yellow substance with abnormal properties.

*o-Methoxybenzylhexamethylenetetraminium chloride.* Melts at  $201-2^\circ$  with decomposition, is readily soluble in water, and gives a deep red color with sulphuric acid.

0.2289 gm. of substance required 7.71 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{14}H_{17}ON_4Cl$ : Cl = 11.95 per cent.

Found: Cl = 11.85 per cent.

*p-Methoxybenzylhexamethylenetetraminium chloride.* Melts at  $180-5^\circ$  with decomposition, is readily soluble in water, and gives a wine-red color with sulphuric acid.



## 674 Salts of Hexamethylenetetramine

0.2027 gm. of substance required 6.85 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{11}\text{H}_{21}\text{ON}_4\text{Cl}$ : Cl = 11.95 per cent.

Found: Cl = 11.89 per cent.

*$\beta$ -Methoxy- $\alpha$ -naphthobenzyl alcohol.* Einhorn and Sprüngerts<sup>22</sup> attempted to convert  $\beta$ -methoxy- $\alpha$ -naphthobenzylamine into the corresponding alcohol, but were able to isolate only a substance of unknown constitution containing nitrogen. We have found that the alcohol may be obtained as follows: 13 grams of the methoxy-naphthobenzylamine were added to a mixture of 150 grams of 30 per cent sodium nitrite solution and 40 grams of acetic acid, causing a vigorous reaction to take place, with the separation of an oil which soon solidified. This was filtered off, washed well with water, and purified by bone-blackening in alcohol, precipitating the substance with water, and repeating the process. Yield: 8.7 grams. For analysis and melting point determination the compound was twice recrystallized from large volumes of water, forming glittering, colorless leaflets, melting at  $100-1^\circ$  (corrected).

0.1144 gm. of substance gave 0.3210 gm.  $\text{CO}_2$  and 0.0678 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_{12}\text{H}_{12}\text{O}_2$ : C = 76.55 per cent; H = 6.43 per cent.

Found: C = 76.53 per cent; H = 6.63 per cent.

*$\beta$ -Methoxy- $\alpha$ -naphthobenzyl chloride.* Partially purified  $\beta$ -methoxy- $\alpha$ -naphthobenzyl alcohol was dissolved in benzene, cooled with ice, and treated with an excess of dry hydrochloric acid gas. After standing for a short time the solution was washed successively with ice water, 5 per cent aqueous sodium carbonate solution, and water, dried over calcium chloride, and evaporated to small bulk. The substance solidified after cooling, and was pressed out on a porous tile and used directly for the preparation of the quaternary salt.

*$\beta$ -Methoxy- $\alpha$ -naphthobenzylhexamethylenetetraminium chloride.* As obtained from chloroform, the salt retained solvent even *in vacuo* at the temperature of boiling alcohol. The chloroform was removed by dissolving the compound in absolute alcohol. Upon addition of dry ether the salt separated as an oil which rapidly solidified. It melts with decomposition at  $185-6^\circ$ , dissolves readily in water, and gives an olive-green color with sulphuric acid.

<sup>22</sup> Einhorn and Sprüngerts: *Ann. d. Chem.*, cccxi, p. 164, 1908.

0.2174 gm. of substance required 6.06 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{12}\text{H}_{11}\text{ON}_4\text{Cl}$ : Cl = 10.23 per cent.

Found: Cl = 9.81 per cent.

*2-Methoxy-5-nitrobenzyl alcohol.* 17 grams of 2-methoxy-5-nitrobenzaldehyde were dissolved in 75 cc. of concentrated methylalcoholic sodium hydroxide, giving rise to a deep red color. After standing over night the mixture was diluted with water to dissolve the precipitated sodium salt of the acid and extracted repeatedly with ether. From this solution a quantitative yield of crude product was obtained. After recrystallization first from toluene and then twice from absolute alcohol, the substance formed rosettes of long, straw-colored needles, melting at  $124-5^\circ$  (corrected), with preliminary softening. The compound is readily soluble in acetone, less so in chloroform and hot water, and difficultly soluble in cold benzene, toluene, and absolute alcohol. The solution in sulphuric acid possesses a brown-orange color.

0.1183 gm. of substance gave 0.2264 gm.  $\text{CO}_2$  and 0.0522 gm.  $\text{H}_2\text{O}$ .

0.1631 gm. of substance (Kjeldahl) required 8.70 cc.  $\frac{\text{N}}{10}$  HCl.

Calculated for  $\text{C}_8\text{H}_9\text{O}_4\text{N}$ : C = 52.44%; H = 4.95%; N = 7.65%.

Found: C = 52.19%; H = 4.94%; N = 7.47%.

*2-Methoxy-5-nitrobenzyl chloride.* The alcohol was boiled for thirty minutes in ten parts of concentrated hydrochloric acid, going into solution at first and then separating as an oil, which crystallized on cooling. After two recrystallizations from absolute alcohol, the chloride formed pale straw-colored prisms, melting at  $80.5-81^\circ$  (corrected), with slight preliminary softening. The substance is very soluble in benzene and chloroform, soluble in ether and absolute alcohol at room temperature, and soluble in sulphuric acid with a bright yellow color.

0.1578 gm. of substance gave 10.05 cc. N (764 mm. and  $29.5^\circ$ ).

0.1553 gm. of substance required 14.32 cc.  $\text{AgNO}_3$  Solution II.<sup>22</sup>

Calculated for  $\text{C}_8\text{H}_9\text{O}_4\text{NCl}$ : N = 6.95 per cent; Cl = 17.60 per cent.

Found: N = 6.94 per cent; Cl = 17.15 per cent.

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<sup>22</sup> After hydrolysis with alcoholic sodium hydroxide, the substance gave a purple color with the indicator. The end-point was easily determined, however, after adding an excess of the silver nitrate solution and boiling until the color was destroyed.

*2-Methoxy-5-nitrobenzylhexamethylenetetraminium chloride.* Melts and decomposes at 191–5°, is very soluble in water, and gives an orange color with sulphuric acid.

0.2295 gm. of substance required 6.68 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>14</sub>H<sub>20</sub>O<sub>3</sub>N<sub>5</sub>Cl: Cl = 10.38 per cent.

Found: Cl = 10.24 per cent.

*3-Nitro-4-methoxybenzyl alcohol.* This substance, already described by Stoermer and Behn,<sup>24</sup> is more conveniently obtained in the following manner: 10 grams of 3-nitroanisaldehyde were dissolved in a small amount of warm methyl alcohol, treated with 50 cc. of saturated methylalcoholic sodium hydroxide, and rapidly chilled. From the deep red solution the sodium salt of the acid separated as a solid cake. If allowed to get too hot, or permitted to stand too long, the alcohol is destroyed. After one hour at 0° enough water was added to dissolve the sodium salt, and the solution repeatedly extracted with ether. Evaporation of this yielded the alcohol practically quantitatively. Recrystallized three times from ether, it formed yellow needles melting at 59–60.5° (corrected), with preliminary softening, and gave an orange color with sulphuric acid.

0.1714 gm. of substance gave 11.8 cc. N (770 mm. and 18.8°).

Calculated for C<sub>8</sub>H<sub>9</sub>O<sub>4</sub>N: N = 7.65 per cent.

Found: N = 7.99 per cent.

*3-Nitro-4-methoxybenzyl chloride.* This was obtained by boiling the alcohol for one-half hour with concentrated hydrochloric acid. Recrystallized twice from absolute alcohol and finally from carbon tetrachloride, it forms drab-colored micro-crystals, melting at 86° (corrected); with preliminary sintering. The substance is soluble in ether, very soluble in benzene, and with difficulty in cold absolute alcohol and carbon tetrachloride.

0.2072 gm. of substance (Kjeldahl) required 10.35 cc.  $\frac{N}{10}$  HCl.

0.2008 gm. of substance gave 0.1414 gm. AgCl.

Calculated for C<sub>8</sub>H<sub>9</sub>O<sub>3</sub>NCl: N = 6.95 per cent; Cl = 17.60 per cent.

Found: N = 7.00 per cent; Cl = 17.42 per cent.

*3-Nitro-4-methoxybenzylhexamethylenetetraminium chloride.* The salt is colorless and melts at 183–8°, with darkening. It is dif-

<sup>24</sup> Stoermer and Behn: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 2459, 1901.

ficultly soluble in water, and gives an orange color with sulphuric acid.

0.2031 gm. of substance required 5.90 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{14}\text{H}_{20}\text{O}_3\text{N}_3\text{Cl}$ : Cl = 10.38 per cent.

Found: Cl = 10.22 per cent.

*o*-Ethoxybenzylhexamethylenetetraminium chloride. The reaction mixture obtained by the use of *o*-ethoxybenzyl chloride<sup>25</sup> was filtered off from a small amount of what was probably hexamethylenetetraminium chloride and treated with several volumes of dry acetone, whereupon the salt crystallized on scratching. It forms microscopic prisms and cubes, melting and decomposing at 188–91°, is very soluble in water, and soluble in sulphuric acid with a rose-red color.

0.2782 gm. of substance required 8.77 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{13}\text{H}_{23}\text{ON}_4\text{Cl}$ : Cl = 11.41 per cent.

Found: Cl = 11.10 per cent.

*3, 4-Methylenedioxybenzylhexamethylenetetraminium chloride.*

The salt, obtained in the usual manner from piperonyl chloride and hexamethylenetetramine, melts and decomposes at 203–4°, is readily soluble in water, and gives a deep purple color with sulphuric acid.

0.1925 gm. of substance required 6.19 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{14}\text{H}_{19}\text{O}_2\text{N}_4\text{Cl}$ : Cl = 11.67 per cent.

Found: Cl = 11.31 per cent.

*2, 3-Dimethoxybenzyl chloride.* Douetteau<sup>26</sup> was unable to obtain this compound in the pure state. It was found, however, that the chloride could be isolated in crystalline form by adding ether to the benzene solution of the crude product and then shaking out successively with ice water, 5 per cent aqueous sodium carbonate, and water, and drying over calcium chloride. The solution was evaporated to small bulk, and the residue taken up in hot ligroin. The needles obtained on cooling were converted into the salt described below, while, for analysis, a portion recovered

<sup>25</sup> Pschorr and Zeidler: *Ann. d. Chem.*, cccclxxiii, p. 76, 1910.

<sup>26</sup> Douetteau: *Bull. Soc. chim.*, series 4, xi, p. 652, 1912.

from the mother liquors was pressed out on a porous plate and recrystallized from absolute alcohol. The substance was then taken up in toluene, filtered from a small amount of impurity, and evaporated to dryness, forming needles melting at 69–70.5° with preliminary softening.

0.2369 gm. of substance required 12.28 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>6</sub>H<sub>11</sub>O<sub>2</sub>Cl: Cl = 19.01 per cent.

Found: Cl = 18.25 per cent.

*2, 3-Dimethoxybenzylhexamethylenetetraminium chloride.* Rhombic crystals, melting at 200–1° with decomposition. The salt is very soluble in water and soluble in sulphuric acid with a deep olive-green color.

0.2137 gm. of substance required 6.48 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>11</sub>H<sub>23</sub>O<sub>2</sub>N<sub>4</sub>Cl: Cl = 10.85 per cent.

Found: Cl = 10.67 per cent.

*2,4-Dimethoxybenzyl alcohol.* Resorcylic aldehyde dimethyl-ether (from resorcylic aldehyde, methyl sulphate, and alkali) was heated on the water bath for two hours with alcoholic potassium hydroxide. On cooling, the resulting mixture was diluted with water and repeatedly extracted with ether. The ethereal solution was dried, evaporated to small bulk, and the residue twice fractionated *in vacuo*. The alcohol boils at 177–9° at 10 mm. and is soluble in hot water, practically insoluble in cold. It dissolves in sulphuric acid with a violet-red color.

0.1215 gm. of substance gave 0.2879 gm. CO<sub>2</sub> and 0.0757 gm. H<sub>2</sub>O.

Calculated for C<sub>7</sub>H<sub>12</sub>O<sub>3</sub>: C = 64.25 per cent; H = 7.20 per cent.

Found: C = 64.62 per cent; H = 6.97 per cent.

*3,4-Dimethoxybenzylhexamethylenetetraminium chloride.* The salt obtained by the use of 3, 4-dimethoxybenzyl chloride<sup>27</sup> was recrystallized from 95 per cent alcohol, and, when dried to constant weight *in vacuo* at room temperature, contained one molecule of water of crystallization and melted at 159–65° with preliminary softening when heated in a tube sealed at both ends. The water was lost *in vacuo* at 100° over phosphoric anhydride, the anhy-

<sup>27</sup> Decker and Pschorr: *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 3404, 1904.

drous substance melting and decomposing at 187–8° with preliminary softening and sintering. It gives a deep purple color with sulphuric acid and is very soluble in water.

0.2000 gm. of substance, dried at room temperature, gave 0.0829 gm. AgCl.

0.5647 gm. of substance, dried at 100°, lost 0.0325 gm.

Calculated for  $C_{15}H_{13}O_2N_4Cl \cdot H_2O$ : Cl = 10.29%;  $H_2O$  = 5.23%.

Found: Cl = 10.26%;  $H_2O$  = 5.76%.

2-Nitro-3,4-dimethoxybenzyl alcohol. This compound, although recently described by Kay and Pictet,<sup>28</sup> had in the meantime been made by us as follows: 10 grams of 2-nitroveratric aldehyde were melted with 7 cc. of absolute alcohol, cooled to 50°, and 10 cc. of saturated alcoholic potassium hydroxide added. The reaction was over in about five minutes, a solid cake of the sodium salt of the acid being formed. After mixing with absolute alcohol, this was filtered off, washed with absolute alcohol, and the filtrate made slightly acid with a solution prepared by the addition of 75 per cent sulphuric acid to absolute alcohol. The resulting potassium sulphate was filtered off, washed with alcohol and ether, and the solution dried over potassium carbonate. The filtrate was evaporated to small bulk *in vacuo*, care being taken to avoid heating. On standing, the residue crystallized, and, after sucking off sharply, was used for the preparation of the chloride. The method outlined above does not give good yields if carried out on a larger scale. The properties of the substance agreed with those recorded by Kay and Pictet.

2-Nitro-3,4-dimethoxybenzyl chloride. This may be more conveniently prepared than by the method of Kay and Pictet by simply boiling the alcohol for five minutes with five to six volumes of concentrated hydrochloric acid. Complete solution does not take place, but the chloride crystallizes immediately on cooling. The greenish product may be recrystallized from ligroin, and then agrees with the description furnished by Kay and Pictet.

2-Nitro-3,4-dimethoxybenzylhexamethylenetetraminium chloride. Glistening leaflets, darkening above 175° and melting at 187–9° with decomposition. The salt is soluble in water with a pale yellow color, and in sulphuric acid with a dull greenish yellow color, darkening on standing.

<sup>28</sup> Kay and Pictet: *Jour. Chem. Soc.*, ciii, p. 947, 1913.

0.2000 gm. of substance required 9.95 cc.  $\text{AgNO}_3$  Solution II.

Calculated for  $\text{C}_{11}\text{H}_{22}\text{O}_4\text{N}_4\text{Cl}$ : Cl = 9.54 per cent.

Found: Cl = 9.26 per cent.

**3-Methoxy-4-ethoxybenzyl alcohol.** 65 grams of ethylvanillin were boiled for one and one-half hours with 240 cc. of saturated alcoholic potassium hydroxide solution. On cooling, the sodium salt of ethylvanillic acid separated. The mixture was treated with two liters of water and extracted several times with ether to remove the 3-methoxy-4-ethoxybenzyl alcohol. After drying and concentrating the extract, the residue was fractionated *in vacuo*. The alcohol boiled at  $185-7^\circ$  at 8 mm. and crystallized in the receiver. The substance agreed in its properties with the description given by Vavon,<sup>29</sup> who obtained the alcohol by the reduction of the aldehyde with platinum black and hydrogen.

**3-Methoxy-4-ethoxybenzyl chloride.** A solution of the alcohol in about ten parts of benzene was saturated at  $0^\circ$  with dry hydrochloric acid and allowed to stand one hour. After shaking out successively with ice water, 5 per cent aqueous sodium carbonate, and water, the solution was dried over calcium chloride, the benzene evaporated off *in vacuo*, and the residue fractionated in a high vacuum. Partial decomposition is apt to occur in the vacuum attainable with the water pump. The chloride boils at about  $127^\circ$  at 0.6–0.7 mm. and solidifies in the receiver to a mass of needles. After recrystallizing twice from dry ether with the aid of a freezing mixture, the chloride softened at  $40^\circ$  and melted at  $42-2.5^\circ$  (corrected). Yield: 6.5 grams, from 10 grams of alcohol. It is readily soluble in the usual organic solvents, gives a bright red color with sulphuric acid, and has a faint, sweet odor.

0.1600 gm. of substance required 14.82 cc.  $\text{AgNO}_3$  Solution II.

Calculated for  $\text{C}_{10}\text{H}_{13}\text{O}_2\text{Cl}$ : Cl = 17.68 per cent.

Found: Cl = 17.22 per cent.

**3-Methoxy-4-ethoxybenzylhexamethylenetetraminium chloride.** As this salt was found to be somewhat soluble in chloroform, the portion obtained from the mother liquors on addition of several volumes of dry acetone was combined with the first fraction and dissolved in a small volume of warm absolute alcohol. After filtering off from the accompanying hexamethylenetetraminium

<sup>29</sup> Vavon: *Compt. rend. Acad. d. sc.*, cliv, p. 360, 1912.

chloride, the salt was recovered from the filtrate by the addition first of dry acetone and then ether. It melts at 178–81° with decomposition and preliminary softening, is easily soluble in water, and gives a wine-red color with sulphuric acid, changing to deep purple on standing.

0.2563 gm. of substance required 7.51 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>14</sub>H<sub>23</sub>O<sub>2</sub>N<sub>4</sub>Cl: Cl = 10.42 per cent.

Found: Cl = 10.31 per cent.

*3-Carboxy-4-oxybenzylhexamethylenetetraminium chloride.* This substance was obtained by heating equimolecular amounts of chloromethylsalicylic acid<sup>30</sup> and hexamethylenetetramine in a sealed tube with dry chloroform for one-half hour, the light yellow salt being purified by boiling out with dry acetone. It forms irregular micro-plates, melting at 188–90° with decomposition, and is fairly soluble, although not completely so, in water, the solution giving a reddish purple color with ferric chloride.

0.2087 gm. of substance gave 0.0889 gm. AgCl.

Calculated for C<sub>14</sub>H<sub>19</sub>O<sub>3</sub>N<sub>4</sub>Cl: Cl = 10.86 per cent.

Found: Cl = 10.53 per cent.

*3-Carbomethoxy-4-oxybenzylhexamethylenetetraminium chloride.* Equimolecular amounts of chloromethylsalicylic acid methyl ester<sup>30</sup> and hexamethylenetetramine were heated with dry chloroform in a sealed tube at 100° for one-half hour, after which the product was pulverized and boiled out with chloroform. The salt melts at 191–1.5° with decomposition, and, in aqueous solution, gives a purple color with ferric chloride.

0.3072 gm. of substance gave 0.1292 gm. AgCl.

Calculated for C<sub>16</sub>H<sub>21</sub>O<sub>3</sub>N<sub>4</sub>Cl: Cl = 10.41 per cent.

Found: Cl = 10.40 per cent.

*2-Oxy-3-carboxy-5-methylbenzylhexamethylenetetraminium chloride.* From chloromethyl-*p*-cresotinic acid,<sup>30</sup> similarly to the salicylic acid compound. Bright yellow powder melting and decomposing at 179–81°. Difficultly and only incompletely soluble in water, the aqueous solution giving a deep violet color with ferric chloride.

<sup>30</sup> D. R. P. 113723. *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, vi, p. 136, 1900–02.



0.2547 gm. of substance gave 0.1071 gm. AgCl.

Calculated for  $C_{18}H_{21}O_3N_4Cl$ : Cl = 10.41 per cent.

Found: Cl = 10.40 per cent.

*2-Oxy-3-carbomethoxynaphthobenzyl chloride.* This was prepared from  $\beta$ -oxynaphthoic acid methyl ester<sup>31</sup> according to D. R. P. 113723,<sup>30</sup> allowing the reaction mixture to stand for one week. The crude product was dried and recrystallized twice from toluene, forming almost colorless woolly masses of delicate needles, melting at 164.5–6° (corrected), with preliminary softening. The compound is readily soluble in the cold in chloroform, less readily in acetone, benzene, and ether, and difficultly in acetic acid and toluene. It gives a greenish yellow color with sulphuric acid.

0.2071 gm. of substance (Carius) gave 0.1181 gm. AgCl.

Calculated for  $C_{18}H_{11}O_3Cl$ : Cl = 14.15 per cent.

Found: Cl = 14.10 per cent.

*2-Oxy-3-carbomethoxynaphthobenzylhexamethylenetetraminium chloride.* The salt separated after a few minutes' boiling of the solution of the components in dry chloroform. The mixture was boiled for one-half hour, most of the chloroform being allowed to evaporate off. The salt contains chloroform of crystallization, which is removed by boiling with dry acetone. It forms faintly yellow micro-crystals which dissolve fairly readily in water, giving a solution which yields an indigo-blue color with ferric chloride. When heated, the substance turns yellow, gradually softens, and decomposes at 195–8°. It turns green in sulphuric acid and dissolves with a greenish yellow color.

0.1681 gm. of substance (Carius) gave 0.0623 gm. AgCl.

Calculated for  $C_{18}H_{23}O_3N_4Cl$ : Cl = 9.08 per cent.

Found: Cl = 9.17 per cent.

*2-Methoxy-5-carboxybenzylhexamethylenetetraminium chloride.* From chloromethylanisic acid<sup>30</sup> and hexamethylenetetramine in dry chloroform at 100° for one-half hour. Melts and decomposes at 204–5° and is very soluble in water, forming a solution that

<sup>31</sup> There seems to be no description of this substance in the literature. Recrystallized from methyl alcohol it forms slightly yellowish prisms which soften above 72° and melt at 73.5–4.° (corrected). In dilute alcohol it gives an indigo-blue color with ferric chloride. The color in sulphuric acid is greenish yellow.

does not become turbid on boiling. It gives a pale brownish yellow solution with sulphuric acid.

0.2079 gm. of substance required 6.02 cc. of  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{18}\text{H}_{21}\text{O}_3\text{N}_4\text{Cl}$ : Cl = 10.41 per cent.

Found: Cl = 10.19 per cent.

*2-Methoxy-5-carbomethoxybenzylhexamethylenetetraminium chloride.* Similarly, from chloromethylanisic acid methyl ester.<sup>30</sup> The salt melts and decomposes at  $208-8.5^\circ$ , is very soluble in water, and gives no color with sulphuric acid.

0.2654 gm. of substance (Kjeldahl) required 30.35 cc.  $\frac{N}{10}$  HCl.

0.2007 gm. of substance required 5.70 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{18}\text{H}_{23}\text{O}_3\text{N}_4\text{Cl}$ : N = 15.81 per cent; Cl = 10.00 per cent.

Found: N = 16.01 per cent; Cl = 9.99 per cent.

*3-Aldehyde-4-oxybenzylhexamethylenetetraminium chloride.* On mixing equimolecular quantities of 5-chloromethylsalicylaldehyde<sup>32</sup> and hexamethylenetetramine and adding dry chloroform, a gummy phenolic salt was immediately formed. On continued boiling, this rearranged into the solid quaternary salt. To facilitate this, the precipitate was pulverized, reunited with the filtrate, and boiled about fifteen minutes longer. The salt forms faintly yellow micro-crystals, decomposing at  $194^\circ$  after turning yellow. It is rather difficultly soluble in water, the solution giving a deep purple color with ferric chloride.

0.2222 gm. of substance gave 0.1017 gm. AgCl.

Calculated for  $\text{C}_{14}\text{H}_{11}\text{O}_2\text{N}_4\text{Cl}$ : Cl = 11.42 per cent.

Found: Cl = 11.32 per cent.

*2-Oxy-3-methoxy-5-aldehydrobenzylhexamethylenetetraminium chloride.* From chloromethylvanillin<sup>32</sup> and hexamethylenetetramine in dry chloroform at  $100^\circ$  in a sealed tube for one-half hour and recrystallization of the product from absolute alcohol. Pale brownish micro-crystals, melting at  $163-4^\circ$  with decomposition. Rather difficultly soluble in water, and gives an orange-brown color with sulphuric acid.

0.2982 gm. of substance gave 0.1216 gm. AgCl.

Calculated for  $\text{C}_{15}\text{H}_{21}\text{O}_3\text{N}_4\text{Cl}$ : Cl = 10.41 per cent.

Found: Cl = 10.08 per cent.

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<sup>32</sup> D. R. P. 114194. *Ibid.*, vi, p. 138, 1900-02.



## THE QUATERNARY SALTS OF HEXAMETHYLENE- TETRAMINE.

### II. MONOHALOGENACETYL BENZYLAMINES AND THEIR HEXA- METHYLENETETRAMINIUM SALTS.

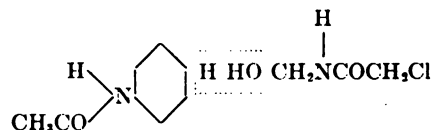
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The present paper deals with a number of chloroacetyl- and iodoacetylbenzylamines and the quaternary salts obtained by their interaction with hexamethylenetetramine.

The halogenacetylbenzylamines were prepared in most cases by the method developed by Einhorn and his collaborators,<sup>1</sup> who found that oxymethylchloroacetamide, under the influence of condensing agents, furnished a valuable means for the introduction of the aminomethyl group into compounds containing the benzene nucleus, chloroacetylbenzylamines being obtained as first products, according to the scheme:



The quaternary salts were prepared by the general method given in the first paper, exceptions being noted as they occurred. In several instances the chloro compounds reacted so unsatisfactorily with hexamethylenetetramine that it was found necessary to convert them into the iodides before good yields of the quaternary salts could be obtained.

<sup>1</sup> Einhorn: *Ann. d. Chem.*, cccxliii, p. 207, 1905; cccclxi, p. 113, 1908.

## EXPERIMENTAL.

*Chloroacetylbenzylamine.* 11 grams of benzylamine were mixed with 100 cc. of normal potassium hydroxide solution, cooled with ice, and treated slowly with 12 gm. of chloroacetyl chloride, with continued shaking and cooling. The substance separated immediately, and was recrystallized from dilute alcohol, forming long, glistening hairs. Recrystallized again from benzene, it melts at 93.5–4.5° (corrected), with slight preliminary softening. It is readily soluble in acetone, and fairly so in cold, dry ether.

0.2312 gm. substance (Kjeldahl) required 12.85 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_9H_{10}ONCl$ : N = 7.63 per cent.

Found: N = 7.78 per cent.

*Chloroacetylbenzylamine and hexamethylenetetramine.* Separation of the salt from the solution obtained by boiling the components in chloroform was facilitated by the addition of an equal volume of warm acetone. It forms microscopic rhombs melting at 168–9° after darkening slightly. It is easily soluble in water and alcohol.

0.2030 gm. of substance required 6.23 cc.  $AgNO_3$  Solution I.<sup>2</sup>

Calculated for  $C_{11}H_{22}ON_4Cl$ : Cl = 10.97 per cent.

Found: Cl = 10.80 per cent.

*Chloroacetyl-o-methylbenzylamine.* Prepared as in the case of the benzyl compound. Long needles from absolute alcohol, easily soluble in the usual solvents, except water and ligroin, and melting at 107.5–8° (corrected).

0.2191 gm. of substance (Kjeldahl) required 11.2 cc.  $\frac{N}{10}$  HCl.

0.1979 gm. of substance required 18.95 cc.  $AgNO_3$  Solution II.<sup>3</sup>

Calculated for  $C_{10}H_{12}ONCl$ : N = 7.09 per cent; Cl = 17.93 per cent.

Found: N = 7.16 per cent; Cl = 17.81 per cent.

*Chloroacetyl-o-methylbenzylamine and hexamethylenetetramine.* The salt was precipitated by evaporating off the chloroform and adding several volumes of dry acetone. It is slowly, although

<sup>2</sup> 1 cc. = 0.00352 gm. Cl; 0.01259 gm. I.

<sup>3</sup> 1 cc. = 0.00186 gm. Cl; 0.00666 gm. I.

freely, soluble in water, and melts at 152° to a yellow liquid which soon decomposes.

0.2089 gm. of substance required 11.30 cc. AgNO<sub>3</sub> Solution II.

Calculated for C<sub>11</sub>H<sub>12</sub>ON<sub>4</sub>Cl: Cl = 10.50 per cent.

Found: Cl = 10.06 per cent.

*p*-Acetaminoiodoacetylbenzylamine. As *p*-acetaminochloroacetylbenzylamine<sup>4</sup> showed no tendency to add to hexamethylenetetramine, the chloro compound was converted into the iodo derivative by boiling in absolute alcohol with 1.5 mols. of sodium iodide for two hours. The mixture was then poured into water and the precipitate recrystallized from hot alcohol by the addition of hot water to faint turbidity. Treated in this way, 12 grams of chloro compound yielded 12.9 grams. The substance, when recrystallized from absolute alcohol, forms glistening needles. When plunged into a bath at 180–90°, it melts with decomposition at 205–6° (corrected), with preliminary darkening. In the cold it is difficultly soluble in the usual organic solvents.

0.2071 gm. of substance (Kjeldahl) required 12.65 cc.  $\frac{N}{10}$  HCl.

0.2060 gm. of substance required 11.63 gm. AgNO<sub>3</sub> Solution II.

Calculated for C<sub>11</sub>H<sub>13</sub>O<sub>2</sub>N<sub>2</sub>I: N = 8.44 per cent; I = 38.20 per cent.

Found: N = 8.55 per cent; I = 37.58 per cent.

*p*-Acetaminoiodoacetylbenzylamine and hexamethylenetetramine. When the two substances were boiled together in dry chloroform alone, there appeared to be no addition, but on adding a concentrated alcoholic solution of hexamethylenetetramine to a chloroform suspension of the iodide, complete solution occurred after ten minutes' boiling. After standing for twenty-four hours in the cold, the long, colorless needles of the salt were filtered off, washed with chloroform, and dried. Yield: about 60 per cent of the theory. The substance could not be obtained in a state of purity, possibly owing to the usually unfavorable effect of the alcohol. The salt is difficultly soluble in water and melts with decomposition at 165–6°.

0.2048 gm. of substance required 4.78 cc. AgNO<sub>3</sub> Solution I.

0.2405 gm. of substance required 5.58 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>17</sub>H<sub>23</sub>O<sub>2</sub>N<sub>6</sub>I: I = 26.88 per cent.

Found: I = 29.39; 29.21 per cent.

<sup>4</sup> Einhorn and Mauermayer: *ibid.*, ccxliii, p. 299, 1905.

*1-Methyl-2-acetaminochloroacetylbenzylamine.* 30 grams of *o*-acettoluidide were dissolved in 200 grams of sulphuric acid, and the solution was well chilled. 25 grams of oxymethylchloroacetamide were then added with vigorous shaking, and the mixture was allowed to stand for two days. After pouring onto ice, the product suddenly crystallized. Recrystallized from 85 per cent alcohol, it melts at  $212.5-4^{\circ}$  (corrected), with slight preliminary softening, the melting point being unchanged by a subsequent recrystallization. The substance is more easily soluble in hot 85 per cent alcohol and hot acetic acid than in the other usual solvents.

0.1489 gm. of substance (Kjeldahl) required 11.7 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_{12}H_{16}O_2N_2Cl$ : N = 11.01 per cent.

Found: N = 11.01 per cent.

*1-Methyl-4-acetaminochloroacetylbenzylamine.* Prepared as in the case of the isomer, letting the reaction mixture stand for three days and recrystallizing the product from 95 per cent alcohol. Recrystallized again from absolute alcohol, it melts incompletely at  $164-7^{\circ}$ , resolidifies again, and melts finally at  $180-2^{\circ}$ .

0.2168 gm. of substance (Kjeldahl) required 17.05 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_{12}H_{16}O_2N_2Cl$ : N = 11.01 per cent.

Found: N = 11.02 per cent.

In an attempt to discover the cause of the peculiar behavior on melting, the substance was heated for ten minutes at  $180-95^{\circ}$ , but only the original substance could be isolated from the melt on cooling.

*1-Methyl-4-acetaminochloroacetylbenzylamine and hexamethylenetetramine.* A suspension of the chloroacetyl compound in a chloroform solution of hexamethylenetetramine was boiled for three hours. The product was filtered off and treated with boiling absolute alcohol in order to remove unchanged material. Only a small amount of the desired salt was left behind. It melts at  $197-200^{\circ}$ .

0.2602 gm. of substance required 6.25 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{13}H_{17}O_2N_4Cl$ : Cl = 8.99 per cent.

Found: Cl = 8.45 per cent.

*$\beta$ -Acetoxy- $\alpha$ -chloroacetylnaphthobenzylamine.* 10 grams of  *$\beta$ -oxy- $\alpha$ -chloroacetylnaphthobenzylamine*<sup>5</sup> were acetylated by warming with 20 cc. of acetic anhydride and a few drops of sulphuric acid. On cooling, the solution set to a thick mass, which was thoroughly shaken with water to decompose the excess of anhydride. After two recrystallizations from absolute alcohol, the substance formed aggregates of long needles, melting at 169–70° (corrected), and readily soluble in cold chloroform, fairly so in cold benzene and acetone, and difficultly soluble in cold ethyl acetate.

0.2439 gm. of substance (Kjeldahl) required 8.55 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_{18}H_{14}O_2NCl$ : N = 4.80 per cent.

Found: N = 4.91 per cent.

*$\beta$ -Acetoxy- $\alpha$ -chloroacetylnaphthobenzylamine and hexamethylene-tetramine.* After one and one-half hours' boiling of the chloroform solution of the components, dry ether was added to incipient turbidity. This resulted in the gradual separation of the crystalline salt, a process which was assisted by the occasional addition of further quantities of ether. The substance was purified by solution in absolute alcohol and reprecipitation with dry ether, after which it darkened at about 170° and melted at 175–6°. It is easily soluble in water.

0.3176 gm. of substance required 7.2 cc.  $AgNO_3$  Solution I.

Calculated for  $C_{21}H_{20}O_2N_4Cl$ : Cl = 8.21 per cent.

Found: Cl = 7.98 per cent.

*$\beta$ -Acetoxy- $\alpha$ -iodoacetylnaphthobenzylamine.* The chloro compound was dissolved by warming in 1.3 molecular equivalents of a normal solution of sodium iodide in dry acetone.<sup>6</sup> The thick, crystalline cake formed after standing for twenty-four hours was broken up, poured into water, and the product recrystallized twice from absolute alcohol. It forms felted needles, fairly soluble in ether and chloroform, but with difficulty in cold absolute alcohol and benzene, and sinters at 187°, melting at 188–90° (corrected) to a brown liquid.

<sup>5</sup> Einhorn and Spröngerts: *ibid.*, cccxi, p. 161, 1908.

<sup>6</sup> Finkelstein: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 1528, 1910.



## 690 Salts of Hexamethylenetetramine

0.2141 gm. of substance (Kjeldahl) required 6.0 cc.  $\frac{N}{10}$  HCl.  
 Calculated for  $C_{15}H_{14}O_2NI$ : N = 3.66 per cent.  
 Found: N = 3.93 per cent.

*$\beta$ -Acetoxy- $\alpha$ -iodoacetylnaphthobenzylamine and hexamethylenetetramine.* The chloroform solution of the components, obtained on gentle heating, was allowed to stand for twenty-four hours and then treated cautiously with dry ether. The salt, which soon separated, was filtered and washed several times with water, in which it is very difficultly soluble. It was then boiled up with dry acetone. It darkens above 175° and melts at 184–7° with decomposition.

0.2008 gm. of substance required 7.07 cc.  $AgNO_3$  Solution II.  
 Calculated for  $C_{21}H_{24}O_2N_4I$ : I = 24.26 per cent.  
 Found: I = 23.43 per cent.

*2-Acetoxy-5-nitrochloroacetylbenzylamine.* The oxy compound<sup>7</sup> was acetylated with acetic anhydride and a few drops of sulphuric acid and recrystallized twice from absolute alcohol. The acetate forms woolly masses of delicate, colorless needles, melting at 97.5–8.° (corrected), with preliminary softening. It is very soluble in chloroform and ether.

0.1493 gm. of substance (Kjeldahl) required 10.45 cc.  $\frac{N}{10}$  HCl.  
 0.0778 gm. of substance gave 0.0403 gm. AgCl.  
 Calculated for  $C_{11}H_{11}O_2N_2Cl$ : N = 9.89 per cent; Cl = 12.37 per cent.  
 Found: N = 9.81 per cent; Cl = 12.82 per cent.

*$\beta$ -Methoxy- $\alpha$ -chloroacetylnaphthobenzylamine<sup>8</sup> and hexamethylenetetramine.* The salt separates with chloroform of crystallization, which is retained even *in vacuo* at the temperature of boiling alcohol. When added to water, it swims about on the surface until dissolved, and evolves enough chloroform to be recognized by its odor.

0.2366 gm. of substance gave 0.0650 gm. AgCl.  
 0.1837 gm. of substance gave 22.0 cc. N (763 mm. and 20.5°).  
 Calculated for  $C_{20}H_{26}O_2N_4Cl \cdot CHCl_3$ : N = 13.39%;  $Cl^-$  = 6.78%.  
 Found: N = 13.65%;  $Cl^-$  = 6.80%.

<sup>7</sup> Einhorn and Mauermayer: *loc. cit.*, p. 286.

<sup>8</sup> Einhorn and Spröngerts: *loc. cit.*, p. 163, preferably using the second method indicated.

The salt, as thus obtained, melts and decomposes at 122.5–5.° when heated in a tube sealed at both ends. The chloroform of crystallization may be removed by boiling the salt about three-quarters of an hour with dry acetone, after which the substance melts at 112–20°. It dissolves slowly, but freely, in water, and gives a deep emerald green color with sulphuric acid.

0.2148 gm. of substance required 5.11 cc. AgNO<sub>3</sub> Solution I.

Calculated: Cl = 8.78 per cent.

Found: Cl = 8.37 per cent.

*2-Methoxy-5-nitrochloroacetylbenzylamine.* Prepared from the oxy compound<sup>7</sup> in the usual manner with aqueous potassium hydroxide and methyl sulphate. Yield: about 40 per cent. The substance was recrystallized first from 50 per cent alcohol, with bone-black, then from absolute alcohol, and finally from chloroform, yielding faintly yellow, minute, glistening prisms, softening at 113–4° and melting at 120.5–1.5° (corrected).

0.1457 gm. of substance (Kjeldahl) required 11.35 cc.  $\frac{N}{10}$  HCl.

0.2483 gm. of substance gave 0.1362 gm. AgCl.

Calculated for C<sub>10</sub>H<sub>11</sub>O<sub>4</sub>N<sub>2</sub>Cl: N = 10.83 per cent; Cl = 13.71 per cent.

Found: N = 10.92 per cent; Cl = 13.57 per cent.

*1-Acetamino-4-ethoxychloroacetylbenzylamine<sup>8</sup> and hexamethylene-tetramine.* The crude quaternary salt was washed well with dry chloroform and acetone and ground up with dry ether to remove an adhering oily impurity. The compound melts with decomposition at 169–70°, is readily soluble in cold water and absolute alcohol, and gives no color with sulphuric acid.

0.2119 gm. of substance required 4.96 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>11</sub>H<sub>21</sub>O<sub>4</sub>N<sub>2</sub>Cl: Cl = 8.35 per cent.

Found: Cl = 8.24 per cent.

*1,2-Diacetoxychloroacetylbenzylamine.* 25 grams of the dioxy compound<sup>10</sup> and 75 cc. of acetic anhydride were treated with a few drops of sulphuric acid. The mixture, which became hot, was further heated for fifteen minutes on the water bath. The solution was cooled and well shaken with water, the resulting oil crystallizing on standing. Yield: 25 grams, after recrystalliza-

<sup>8</sup> Einhorn and Mauermayer: *loc. cit.*, p. 301.

<sup>10</sup> Einhorn and Mauermayer: *loc. cit.*, p. 290.

tion from dilute alcohol. Recrystallized successively from absolute alcohol, toluene, and absolute alcohol, the substance melted at 86.5–7.5° (corrected), with preliminary softening. It forms sheaves of glistening, flat rods, easily soluble in chloroform and less soluble in ether.

0.1956 gm. of substance (Kjeldahl) required 6.6 cc.  $\frac{N}{16}$  HCl.  
 Calculated for  $C_{13}H_{14}O_2NCl$ : N = 4.67 per cent.  
 Found: N = 4.73 per cent.

*1,2-Diacetoxychloroacetylbenzylamine and hexamethylenetetramine.* The salt dissolves readily in water and melts with decomposition at 174°.

0.2663 gm. of substance required 5.95 cc. AgNO<sub>3</sub> Solution I.  
 Calculated for  $C_{13}H_{14}O_4N_2Cl$ : Cl = 8.06 per cent.  
 Found: Cl = 7.87 per cent.

*1,2-Dimethoxychloroacetylbenzylamine.* The dioxy compound was methylated in the usual manner by the use of aqueous potassium hydroxide and methyl sulphate. Recrystallized twice from absolute alcohol, then toluene, and finally again from absolute alcohol, the substance melts at 117–7.5° (corrected), with preliminary softening. It forms cream-colored masses of delicate needles which dissolve in chloroform, are less soluble in benzene, and give a faint yellowish color in sulphuric acid.

0.1965 gm. of substance (Kjeldahl) required 8.25 cc.  $\frac{N}{16}$  HCl.  
 Calculated for  $C_{11}H_{14}O_2NCl$ : N = 5.75 per cent.  
 Found: N = 5.88 per cent.

*1,2-Dimethoxychloroacetylbenzylamine and hexamethylenetetramine.* After one and one-half hours' boiling, the salt was precipitated by means of dry acetone, forming slightly brownish microcrystals, dissolving readily in water, and melting at 160–1° with decomposition. The salt dissolves in sulphuric acid with a violet-red color and violet fluorescence.

0.3139 gm. of substance required 8.11 cc. AgNO<sub>3</sub> Solution I.  
 Calculated for  $C_{17}H_{26}O_2N_2Cl$ : Cl = 9.24 per cent.  
 Found: Cl = 9.09 per cent.

*m-Chloroacetylaminomethylbenzoic acid ethyl ester (m-carbethoxychloroacetylbenzylamine)<sup>11</sup> and hexamethylenetetramine.* The solu-

<sup>11</sup> Einhorn and Mauermayer: *loc. cit.*, p. 296.

tion obtained by boiling the two substances in chloroform for one-half hour was concentrated to small bulk and treated with several volumes of dry acetone. The salt crystallized on scratching, and melted at 122–4°. It is easily soluble in cold absolute alcohol, chloroform and water, and fairly soluble in hot acetone.

0.2017 gm. of substance required 5.0 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{11}\text{H}_{11}\text{O}_2\text{N}_2\text{Cl}$ : Cl = 8.96 per cent.

Found: Cl = 8.73 per cent.

*m*-Iodoacetylaminomethylbenzoic acid ethyl ester. This was prepared from the chloro compound by the use of sodium iodide in acetone. Recrystallized successively from absolute alcohol, toluene, and absolute alcohol, it forms glistening, delicate needles, melting at 116–6.5° (corrected), with preliminary softening, and dissolving with great difficulty at 0° in ether, absolute alcohol, and toluene, and readily in chloroform.

0.2860 gm. of substance (Kjeldahl) required 8.4 cc.  $\frac{\text{N}}{10}$  HCl.

Calculated for  $\text{C}_{12}\text{H}_{14}\text{O}_2\text{NI}$ : N = 4.04 per cent.

Found: N = 4.11 per cent.

*m*-Chloroacetylaminomethylbenzoyl chloride. Equal weights of the acid,<sup>12</sup> toluene, and phosphorus pentachloride were heated on the water bath until evolution of hydrochloric acid ceased. The green solution was then evaporated to dryness *in vacuo*, more toluene added, and the solvent again evaporated off. On dissolving in hot toluene and adding an equal volume of ligroin the chloride separated in long needles. Yield: equal to the amount of acid taken. Recrystallized from a small volume of toluene, it forms aggregates of pale greenish prisms, melting at 78–9° (corrected), with slight preliminary softening.

Hydrolysis in the cold with alcoholic ammonia:

0.1669 gm. of substance required 6.75 cc.  $\text{AgNO}_3$  Solution I.

0.1884 gm. of substance required 7.75 cc.  $\text{AgNO}_3$  Solution I.

Calculated for  $\text{C}_{10}\text{H}_9\text{O}_2\text{NCl}_2$ : Cl<sub>1</sub> = 14.41 per cent.

Found: Cl<sub>1</sub> = 14.25; 14.47 per cent.

*m*-Chloroacetylaminomethylbenzoic acid diethylaminoethyl ester. 27 grams of the chloride were dissolved in dry chloroform and added, with chilling, to a solution of 13 grams of diethylaminoethanol in 75 grams of pyridine. After standing for one hour, the solution was diluted with water and concentrated at room

<sup>12</sup> Einhorn and Mauermayer: *loc. cit.*, p. 295.

temperature *in vacuo* to a thick syrup. This was taken up with water and the ester precipitated with aqueous sodium carbonate as an oil which solidified on scratching and chilling. The substance was purified by dissolving in dilute hydrochloric acid and again precipitating with sodium carbonate. Yield: 18 grams. Recrystallized from ligroin, it forms glistening platelets, sintering at 68° and melting at 69–70.5° (corrected).

0.1997 gm. of substance required 6.1 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>14</sub>H<sub>21</sub>O<sub>2</sub>N<sub>2</sub>Cl: Cl = 10.86 per cent.

Found: Cl = 10.75 per cent.

*m*-Chloroacetylaminomethylbenzoic acid diethylaminoethyl ester and hexamethylenetetramine. The addition of dry ether to the chloroform reaction mixture was necessary to cause crystallization. Rapid manipulation was essential, as the salt proved to be very hygroscopic. It is easily soluble in water, alcohol, chloroform, and acetone. It sinters at 120° and melts at 125–30°.

0.2290 gm. of substance required 5.2 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>27</sub>H<sub>35</sub>O<sub>2</sub>N<sub>4</sub>Cl: Cl = 7.61 per cent.

Found: Cl = 7.99 per cent.

*m*-Chloroacetylaminomethylbenzamide. 22 grams of chloride were dissolved in dry chloroform, cooled in a freezing mixture, and, with continuous stirring, treated with an excess of concentrated aqueous ammonia, added drop by drop. The amide separated at once, and was recrystallized from water. Yield: 17 grams. After boiling with acetone to remove the yellow color, and recrystallizing from amyl alcohol, the substance melted at 171–2.5° (corrected), with slight preliminary softening.

0.1746 gm. of substance (Kjeldahl) required 15.5 cc.  $\frac{N}{16}$  HCl.

Calculated for C<sub>10</sub>H<sub>11</sub>O<sub>2</sub>N<sub>2</sub>Cl: N = 12.37 per cent.

Found: N = 12.44 per cent.

*m*-Chloroacetylaminomethylbenzamide and hexamethylenetetramine. 7 grams of amide and 4.5 grams of base were boiled for several hours in 600 cc. of acetone, and the salt was filtered off. An additional amount was obtained from the filtrate on longer boiling. The substance darkens above 175°, melts with decomposition at 183°, and is readily soluble in water.

0.2159 gm. of substance required 5.75 cc. AgNO<sub>3</sub> Solution I.

Calculated for C<sub>16</sub>H<sub>23</sub>O<sub>2</sub>N<sub>6</sub>Cl: Cl = 9.67 per cent.

Found: Cl = 9.38 per cent.

## ON THE PRESENCE OF IODINE IN THE HUMAN FETAL THYROID GLAND.

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It does not seem reasonable that the human fetal thyroid should be without iodine when we have conclusive evidence of its presence in fetuses from domestic animals.<sup>1</sup> Cameron<sup>2</sup> believes that the negative figures for new-born children's thyroids recorded by previous investigators were due simply to the less accurate analytical methods available at the time, and that the small amount of iodine actually present escaped detection. The present work proves this assumption to be correct.

This investigation was conducted on glands from ten human fetuses, five males and five females, six to nine months old. The thyroids were removed from the fetuses, freed from connective and other tissue, weighed, finely minced, and dried to constant weight. Extraction with petroleum ether was omitted as the desiccated material was practically free from fat. The larger glands were of a dark reddish brown color while the smaller glands were light colored. In the tabulation are given the maximum, minimum, and average weights of both male and female glands, also the moisture in the fresh glands, together with the iodine, phosphoric acid, and ash contents of the desiccated substance.

The iodine was determined according to the Hunter method,<sup>3</sup> while the methods employed for the ash and phosphoric acid determinations have been described elsewhere,<sup>4</sup> in connection with some previous investigations on the thyroids from domestic animals.

<sup>1</sup> F. Fenger: this *Journal*, xi, p. 489, 1912; xii, p. 55, 1912; xvii, p. 23, 1914.

<sup>2</sup> A. T. Cameron: *ibid.*, xvi, pp. 465-473, 1913-14.

<sup>3</sup> A. Hunter: *ibid.*, vii, pp. 321-349, 1909-10.

<sup>4</sup> A. Seidell and F. Fenger: *Bull. Hyg. Lab., U. S. P. H. and M.-H. S.*, 96, 1914.

## 696 Iodine in the Human Fetal Thyroid Gland

It will be noticed that glands from both sexes contain iodine, the quantity being very small, however, especially in the case of the female. The size of the thyroid gland is, as a rule, in inverse proportion to its iodine content. Logically, therefore, we should expect to find a much smaller percentage of iodine in the larger female glands than in the normal sized male glands, as is the case. The phosphorus content of the female glands is higher than that in the male glands.

TABLE I.

	TOTAL NO. OF GLANDS	MAXIMUM WEIGHT OF GLANDS	MINIMUM WEIGHT OF GLANDS	AVERAGE WEIGHT OF GLANDS	MOISTURE IN FRESH GLANDS	IODINE IN DESIC- CATED GLANDS	PO <sub>4</sub> IN DESIC- CATED GLANDS	ASH IN DESIC- CATED GLANDS
		gm.	gm.	gm.	per cent	per cent	per cent	per cent
Male fetal thyroids	5	3.1	1.1	2.6	78.0	0.029	1.68	5.20
Female fetal thyroids	5	9.2	1.5	4.5	71.9	0.005	1.84	5.30

The fact that the female fetal glands, in this particular instance, are larger than the male thyroids, is undoubtedly an accidental coincidence, and the difference in the quantity of iodine between the male and female glands should not be considered as specific or characteristic of the two sexes. It has been shown elsewhere<sup>5</sup> that a certain percentage of beef fetuses of both sexes contains enlarged thyroids very low in iodine and proportionally high in phosphorus content. Similar conditions evidently exist in human fetuses.

### CONCLUSIONS.

It has been shown conclusively that both enlarged and normal sized human fetal thyroids contain iodine at least during the last three months of intra-uterine life.

Normal sized fetal glands contain relatively more iodine and less phosphorus than enlarged fetal glands. These conditions are analogous to those existing in the fetal and adult thyroids from cattle, hogs, and sheep.

<sup>5</sup> Fenger: this *Journal*, xvii, p. 23, 1914.

## ON ALFALFA LACCASE.<sup>1</sup>

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(Received for publication, February 12, 1915.)

### I. INTRODUCTION.

In a series of articles<sup>2</sup> Euler and Bolin came to the conclusion that *Medicago laccase*, an oxidase first prepared by Bertrand<sup>3</sup> in 1897, is simply a mixture of the calcium salts of mono-, di-, and tri-basic organic acids, particularly citric, malic, mesoxalic, and probably glyoxalic acids. This statement created a great deal of interest among plant physiologists and biological chemists; it was the first instance of the recognition of the chemical composition of an oxidase. It was naturally widely heralded through scientific articles<sup>4</sup> and text-books.<sup>5</sup>

The experimental facts which Euler and Bolin present in substantiation of their claim are the following:

1. In solutions weakly acid to phenolphthalein containing very low concentrations of manganese acetate, Bertrand's *Medicago*

<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> H. Euler and I. Bolin: Zur Kenntnis biologisch wichtiger Oxydationen, *Ztschr. f. physiol. Chem.*, lvii, pp. 80-98, 2 fig., 1908; lxi, pt. i, pp. 1-11, 72-92, 1909; Über die chemische Zusammensetzung und die biologische Rolle einer Oxydase, *Ztschr. f. physikal. Chem.*, lxi, pp. 187-202, 2 fig., 1909.

<sup>3</sup> G. Bertrand: Sur l'intervention du manganèse dans les oxydations provoquées par la laccase, *Bull. Soc. chim.*, series 3, xvii, p. 621, 1897.

<sup>4</sup> F. Battelli and L. Stern: Die Oxydationsfermente, *Ergebn. d. Physiol.*, xii, p. 242, 1912.

<sup>5</sup> O. von Fürth: *Probleme der physiologischen und pathologischen Chemie*, Leipsic, 1913, ii, p. 536. H. Euler: *General Chemistry of the Enzymes*, translated from the revised German edition by Thomas H. Pope, 1st edition, New York, 1912, p. 222. E. Abderhalden: *Biochemisches Handlexikon*, v, p. 639, 1911.



*laccase* greatly accelerates the oxidation of hydroquinone by atmospheric oxygen.

2. In solutions weakly acid to phenolphthalein, sodium potassium tartrate, sodium succinate, sodium fumarate, sodium lactate, sodium acetate, sodium citrate, sodium mucinate, and calcium gluconate accelerate the oxidation of hydroquinone by atmospheric oxygen to approximately the same extent as equal concentrations of *Medicago laccase*.

3. Laccase preparations made according to a method somewhat similar to Bertrand's are a mixture of calcium citrate, calcium malate, calcium mesoxalate, and calcium glycolate.

The existence of a laccase as an oxidase in the sense used by Euler and Bolin becomes uncertain, through the work of Ruff,<sup>6</sup> Trillat,<sup>7</sup> Dony-Hénault,<sup>8</sup> and Fouard.<sup>9</sup> According to Dony-Hénault there is no laccase in the sense in which Bertrand uses the word. Manganous or ferrous salts together with colloids in alkaline solutions produce the same effect. The concentration of the hydroxyl ions needs to be only slightly greater than in neutral solutions, inasmuch as the oxidation of hydroquinone will go on quite rapidly in slightly alkaline aqueous solutions. Sjollemma<sup>10</sup> comes to similar conclusions. He finds that colloidal solutions formed by manganous sulphate or acetate with sodium potassium tartrate, hydrogen peroxide, and sodium hydrate exhibit a behavior like that of laccase. Sjollemma attributes this behavior to the higher oxides of manganese present in such solutions. In view of the well known fact that hydroquinone is rapidly oxidized

<sup>6</sup> C. Oppenheimer: *Die Fermente und ihre Wirkungen*, 2d edition, Leipsic, 1903, p. 368.

<sup>7</sup> A. Trillat: Influence activante d'une matière albuminoïde sur l'oxydation provoquée par le manganèse, *Compt. rend. Acad. d. sc.*, cxxxviii, pp. 94-96, 1904; Sur le rôle d'oxydases que peuvent jouer les sels manganoux en présence d'un colloïde, *ibid.*, cxxxviii, pp. 274-77, 1904.

<sup>8</sup> O. Dony-Hénault: Contribution à l'étude méthodique des oxydases, *Acad. roy. Belg., Bull. cl. Sc.*, pp. 105-163, 1908. O. Dony-Hénault and J. van Duuren: Contribution à l'étude méthodique des oxydases dans les tissus animaux, *ibid.*, pp. 537-638, 1907.

<sup>9</sup> E. Fouard: Sur l'action catalytique exercée par les sels alcalins et alcalino-terreux dans la fixation de l'oxygène de l'air par les solutions de polyphénols, *Compt. rend. Acad. d. sc.*, cxlii, pp. 796-798, 1906.

<sup>10</sup> B. Sjollemma: Over de beteekenis van colloïdale mangaanoxydoplossingen bij biochemische oxydaties, *Chem. Weekbl.*, vi, pp. 287-294, 1909.

in very weakly alkaline solutions even in the absence of manganese salts and oxidases, it seemed desirable to repeat the experiments of Euler and Bolin with a careful control of the hydrogen ion concentrations.

## II. EXPERIMENTAL.

The rates of oxidation were determined by the formerly described methods.<sup>11</sup> The small apparatus<sup>12</sup> with a capacity of 25 cc. was used. The total volume of liquid in all experiments was 6 cc., leaving the total volume of air in the apparatus 19 cc. Inasmuch as all of the experiments were strictly comparative, the actual manometer readings are given. The temperature of the box in which the experiments were carried out was 37.4°C., and the rate of shaking eighty-eight complete excursions per minute. The latitude of the excursions was 10 cm. The hydrogen ion determinations were made by the gas chain method, by the use of an  $\frac{N}{10}$  calomel electrode to complete the chain. For the sake of maintaining the concentration of the hydrogen ions constant throughout the experiment, Henderson's<sup>13</sup> balanced phosphate solutions were used. Before the solutions were used, their hydrogen ion concentration was determined by means of the gas chain method. For a standard of comparison, the absorption expressed in terms of centimeters of mercury produced in the course of forty-five minutes was chosen.

<sup>11</sup> H. H. Bunzel: The measurement of the oxidase content of plant juices, *U. S. Dept. Agriculture, Bureau of Plant Industry, Bull. 238*, 40 pp., 9 fig., 2 pl., 1912. Also published in condensed form in *Jour. Am. Chem. Soc.*, xxxiv, pp. 303-317, 2 fig., 1912.

<sup>12</sup> Bunzel: A simplified and inexpensive oxidase apparatus, this *Journal*, xvii, pp. 409-411, 1 fig., 1914.

<sup>13</sup> L. J. Henderson and O. F. Black: Concerning the neutrality of protoplasm, *Am. Jour. Physiol.*, xviii, pp. 250-255, 1907. A study of the equilibrium between carbonic acid, sodium bicarbonate, mono-sodium phosphate, and di-sodium phosphate at body temperature, *ibid.*, xxi, pp. 420-426, 2 fig., 1908.

*Series 1.*

*The rate of oxidation of hydroquinone in solutions containing various concentrations of hydrogen ions.*

In each one of these experiments 0.01 gm. of hydroquinone in 6 cc. of solution was used. The desired hydrogen ion concentrations were obtained by the use of different proportions of molecular orthophosphoric acid and normal sodium hydrate solution. In all of these experiments 3 cc. of the phosphate mixture and 3 cc. of water were used.

CONCENTRATION OF HYDROGEN IONS	MANOMETER READINGS AFTER 45 MIN.
	cm.
$1.1 \times 10^{-7}$	0.00
$5.6 \times 10^{-8}$	0.55
$2.8 \times 10^{-8}$	2.38
$1.4 \times 10^{-8}$	4.20
$7.0 \times 10^{-9}$	7.05

*Series 2.*

*The rate of oxidation of hydroquinone in solutions containing sodium succinate.*

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF SODIUM SUCCINATE	CONCENTRATION* OF HYDROGEN IONS	MANOMETER READINGS AFTER 45 MIN.
			cm.
0.03 N	0.167 N	$4.5 \times 10^{-8}$	1.15
0.03 N	0.333 N	$4.0 \times 10^{-8}$	2.00
0.03 N	0.500 N	$2.2 \times 10^{-8}$	2.60
0.03 N	0.667 N	$2.0 \times 10^{-8}$	2.88

\*Considerable difficulty was experienced in determining the hydrogen ion concentrations by the gas chain method in this series. This is probably due to the difficulty in keeping the platinum electrode saturated with hydrogen. In alkaline solutions the oxidation of hydroquinone is comparatively rapid, and the product of oxidation would tend to oxidize the hydrogen on the platinum electrode. In this series, therefore, the determinations were made colorimetrically. Tropaeolin 000 I served as indicator; the color standards were prepared by means of Henderson's phosphate mixtures.

*Series 3.*

*The rate of oxidation of hydroquinone in solutions containing sodium succinate and phosphate mixtures.*

In this series of experiments 3 cc. of the various phosphate mixtures were used with either 3 cc. of the normal sodium succinate solution or with 3 cc. of water.

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF SODIUM SUCCINATE	CONCENTRATION OF HYDROGEN IONS	MANOMETER READINGS AFTER 45 MIN.
			cm.
0.03 N	.....	$1.1 \times 10^{-7}$	0
0.03 N	0.5 N	$1.1 \times 10^{-7}$	0
0.03 N	.....	$5.6 \times 10^{-8}$	0.30
0.03 N	0.5 N	$5.6 \times 10^{-8}$	0.40
0.03 N	.....	$2.8 \times 10^{-8}$	2.45
0.03 N	0.5 N	$2.8 \times 10^{-8}$	?
0.03 N	.....	$1.4 \times 10^{-8}$	4.65
0.03 N	0.5 N	$1.4 \times 10^{-8}$	4.60
0.03 N	.....	$7.0 \times 10^{-9}$	6.00
0.03 N	0.5 N	$7.0 \times 10^{-9}$	6.00

*Series 4.*

*Rate of oxidation of hydroquinone in neutral solutions containing various concentrations of manganese acetate.*

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF HYDROGEN IONS	CONCENTRATION OF MANGANESE ACETATE	MANOMETER READINGS AFTER 45 MIN.
			cm.
0.03 N	$1.1 \times 10^{-7}$	.....	0
0.03 N	$1.1 \times 10^{-7}$	0.0008 N	0.30
0.03 N	$1.1 \times 10^{-7}$	0.0017 N	0.30
0.03 N	$1.1 \times 10^{-7}$	0.0033 N	0.60
0.03 N	$1.1 \times 10^{-7}$	0.0050 N	0.70

*Series 5.*

*Rate of oxidation of hydroquinone in alkaline solutions ( $C_H = 5.6 \times 10^{-8}$ ) containing various concentrations of manganese acetate.*

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF HYDROGEN IONS	CONCENTRATION OF MANGANESE ACETATE	MANOMETER READINGS AFTER 45 MIN.
			cm.
0.03 N	$5.6 \times 10^{-8}$	0	0.78
0.03 N	$5.6 \times 10^{-8}$	0.0008 N	1.82
0.03 N	$5.6 \times 10^{-8}$	0.0017 N	2.05
0.03 N	$5.6 \times 10^{-8}$	0.0033 N	2.05
0.03 N	$5.6 \times 10^{-8}$	0.0050 N	2.45

## Series 6.

*Rate of oxidation of hydroquinone in acid solutions ( $C_H = 2.3 \times 10^{-7}$ ) containing various concentrations of manganese acetate.*

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF HYDROGEN IONS	CONCENTRATION OF MANGANESE ACETATE	MANOMETER READINGS AFTER 45 MIN.
			cm.
0.03 N	$2.3 \times 10^{-7}$	0	0
0.03 N	$2.3 \times 10^{-7}$	0.0008 N	0.30
0.03 N	$2.3 \times 10^{-7}$	0.0017 N	0.35
0.03 N	$2.3 \times 10^{-7}$	0.0033 N	0.40
0.03 N	$2.3 \times 10^{-7}$	0.0050 N	0.70
0.03 N	$2.3 \times 10^{-7}$	0.0067 N	1.60

## Series 7.

*Rate of oxidation of hydroquinone in acid solutions ( $C_H = 4.5 \times 10^{-7}$ ) containing various concentrations of manganese acetate.*

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF HYDROGEN IONS	CONCENTRATION OF MANGANESE ACETATE	MANOMETER READINGS AFTER 45 MIN.
			cm.
0.03 N	$4.5 \times 10^{-7}$	0	0
0.03 N	$4.5 \times 10^{-7}$	0.0008 N	0
0.03 N	$4.5 \times 10^{-7}$	0.0017 N	0.10
0.03 N	$4.5 \times 10^{-7}$	0.0033 N	0.30
0.03 N	$4.5 \times 10^{-7}$	0.0050 N	0.50

## Series 8.

*Rate of oxidation of hydroquinone in neutral solutions containing various quantities of Medicago sativa powder.\**

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF HYDROGEN IONS	CONCENTRATION OF MEDICAGO SATIVA	MANOMETER READINGS AFTER 45 MIN.
		per cent	
0.03 N	$1.1 \times 10^{-7}$	.....	0
0.03 N	$1.1 \times 10^{-7}$	0.167	0
0.03 N	$1.1 \times 10^{-7}$	0.333	0
0.03 N	$1.1 \times 10^{-7}$	0.666	0
0.03 N	$1.1 \times 10^{-7}$	1.000	0

\*Leaves collected from flowering alfalfa grown in the greenhouse were dried *in vacuo* over lime, powdered, and sifted through a 100 mesh to the inch sieve. Only the fine powder was used.

## Series 9.

*Rate of oxidation of hydroquinone in aqueous solutions containing various quantities of Medicago sativa leaf powder.*

CONCENTRATION OF HYDROQUINONE	CONCENTRATION OF MEDICAGO SATIVA	MANOMETER READINGS AFTER 45 MIN.
	<i>per cent</i>	
0.03 N	.....	0
0.03 N	0.167	0
0.03 N	0.333	0
0.03 N	0.666	0
0.03 N	1.000	-0.10

## Series 10.

*Rate of oxidation of hydroquinone in aqueous solutions containing various concentrations of manganese acetate and a fixed quantity of Medicago sativa leaf powder.*

CONCENTRA- TION OF HYDROQUI- NONE	CONCENTRATION OF HYDROGEN IONS	CONCENTRATION OF MANGANESE ACETATE	CONCENTRATION OF MEDICAGO SATIVA	MANOMETER READINGS AFTER 45 MIN.
			<i>per cent</i>	<i>cm.</i>
0.03 N	$1.1 \times 10^{-7}$	0	0.167	0
0.03 N	$1.1 \times 10^{-7}$	0.0008 N	0.167	0
0.03 N	$1.1 \times 10^{-7}$	0.0017 N	0.167	0.25
0.03 N	$1.1 \times 10^{-7}$	0.0033 N	0.167	0.35
0.03 N	$1.1 \times 10^{-7}$	0.0050 N	0.167	0.80

## Series 11.

*Rate of oxidation of hydroquinone in aqueous solutions containing various quantities of Medicago sativa powder and a fixed concentration of manganese acetate.*

CONCENTRA- TION OF HYDROQUI- NONE	CONCENTRATION OF HYDROGEN IONS	CONCENTRATION OF MANGANESE ACETATE	CONCENTRATION OF MEDICAGO SATIVA	MANOMETER READINGS AFTER 45 MIN.
			<i>per cent</i>	<i>cm.</i>
0.03 N	$1.1 \times 10^{-7}$	0.0017 N	0	0
0.03 N	$1.1 \times 10^{-7}$	0.0017 N	0.167	0.17
0.03 N	$1.1 \times 10^{-7}$	0.0017 N	0.333	0.10
0.03 N	$1.1 \times 10^{-7}$	0.0017 N	0.500	0.15
0.03 N	$1.1 \times 10^{-7}$	0.0017 N	0.666	0.20

## III. DISCUSSION.

On the basis of these experiments, it becomes evident that the alfalfa leaf powder has no measurable effect on the rate of oxidation in the presence or absence of manganese acetate. Euler and Bolin came to a different conclusion. They found that traces of manganese salts accelerated markedly the oxidation of hydroquinone by alfalfa laccase. But the laccase preparations of Euler and Bolin, as they themselves show, consist simply of salts of strong bases, principally calcium, with weak organic acids. From our knowledge of the behavior of salts of strong bases, and weak acids, in solution, it may be concluded that a solution of such salts would be alkaline. As a matter of fact, Series 2 shows that the concentration of hydrogen ions in a 0.167 N solution of sodium succinate containing 0.01 gram of hydroquinone in 6 cc. of solution is  $4.5 \times 10^{-8}$ , and that in a solution of that alkalinity the rate of oxidation is fairly rapid. Euler and Bolin claim that they were very careful in the experiments relating to the effect of laccase to maintain their solutions slightly acid. They used phenolphthalein as indicator. Phenolphthalein will indicate an acid reaction, *i.e.*, it will remain colorless in solutions having a concentration of hydrogen ions<sup>14</sup> of  $3 \times 10^{-8}$  or greater. Series 1 shows that in solutions having a  $C_H$  of  $3 \times 10^{-8}$  the rate of oxidation is quite rapid. It is therefore easily possible to prepare solutions which contain hydrogen ions in such concentration that the solutions do not color phenolphthalein, and in which the concentration of hydroxyl ions is still sufficiently great to bring about a rapid oxidation of hydroquinone. A very clear insight into this fact may be obtained from Figure 1. Here the rates of oxidation of hydroquinone in solutions of different acidity were plotted. The heavy section of the curve represents the range of concentrations of hydrogen ions within which oxidation proceeds at a rate measurable under the experimental conditions, and within which the concentration of the hydrogen ions is sufficiently great to prevent the coloration of the phenolphthalein indicator.

Quite in accordance with Euler and Bolin's results, hydroxyl ions even in such concentrations as are present in solutions neutral to

<sup>14</sup> W. Nernst: *Theoretical Chemistry*, revised in accordance with the 6th German edition by H. T. Tizard, 810 pp., 50 fig., London, 1911.

phenolphthalein bring about a fairly rapid oxidation of hydroquinone. With increasing concentrations of hydroxyl ions, the rate of oxidation is, of course, very much increased. In view of the fact that hydroquinone is a weak acid,<sup>15</sup> it is likely that the conditions for its oxidation are the same as those determining the oxidation of glucose, as shown by Mathews and the writer<sup>16</sup> a number of years ago.

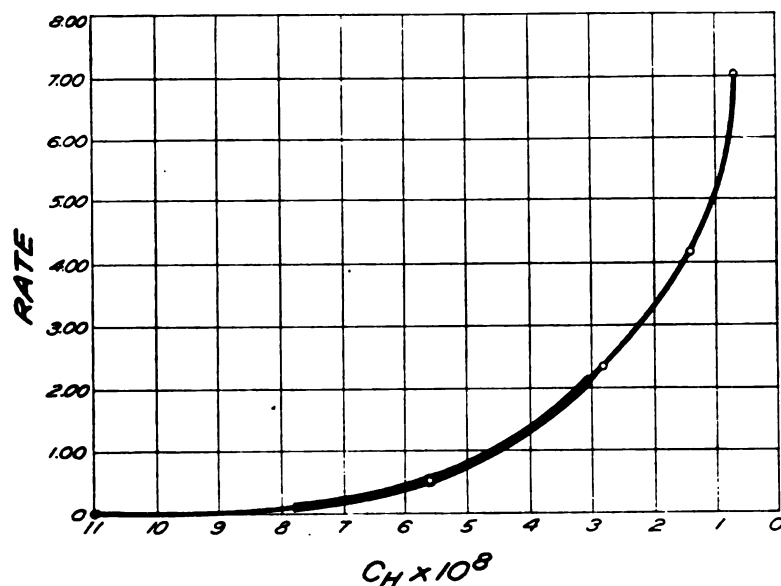


FIG. 1. Curve showing the rate of oxidation of hydroquinone in solutions containing various concentrations of hydrogen ions.

The observations of Euler and Bolin that the sodium salts of organic acids accelerate the oxidation of hydroquinone by atmospheric oxygen were confirmed in the case of sodium succinate. It is well known that all such salts are, according to the strength of the acid, more or less hydrolyzed. The weaker the acid, the

<sup>15</sup> H. Scudder: *The Electrical Conductivity and Ionization Constants of Organic Compounds*, 179 pp., New York, 1914.

<sup>16</sup> H. H. Bunzel and A. P. Mathews: The mechanism of the oxidation of glucose by bromine in neutral and acid solutions, *Jour. Am. Chem. Soc.*, xxi, pp. 464-479, 1 fig., 1909. H. H. Bunzel: The mechanism of the oxidation of glucose by bromine, *this Journal*, vii, pp. 157-169, 1909-10.



greater the degree of hydrolysis. It occurred to the writer that the accelerating effect of the salts tried by Euler and Bolin might be due simply to the hydroxyl ions which they furnish when dissolved in water. This supposition was found to be true. Series 2 shows the rate of oxidation of hydroquinone in solutions containing various concentrations of sodium succinate. If the rates of oxidation in these solutions are compared with the rates in solutions containing the same number of hydroxyl ions, it is found that the rates of oxidation in the two solutions are the same. In other words, the rates of oxidation in solutions containing the same concentrations of hydroxyl ions are equal, no matter whether the particular concentration of hydroxyl ions is brought about by mixtures of mono-sodium phosphate and di-sodium phosphate or by the hydrolysis of sodium succinate and the dissociation of the sodium hydrate thus formed. Moreover, when sodium succinate is added to solutions of hydroquinone in which solutions the concentration of hydrogen ions is maintained constant by balanced phosphate mixtures, no effect is produced on the rate of oxidation.

#### IV. SUMMARY.

1. The effect of salts of strong bases with weak acids on the rate of oxidation of hydroquinone by atmospheric oxygen is due entirely to the concentrations of the hydroxyl ions in such solutions.
2. There is no hydroquinone-oxidizing oxidase "laccase" in *Medicago sativa*.
3. The accelerating effect of Euler and Bolin's "laccase" prepared from *Medicago sativa* on the rate of oxidation of hydroquinone by atmospheric oxygen is due to the alkalinity of the solutions of the salts contained in their preparations.

## HUMAN MILK.

By ALFRED W. BOSWORTH.

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(Received for publication, February 19, 1915.)

### INTRODUCTION.

The knowledge of the chemistry of human milk has been derived from a general study of the constituents present and may be summarized by a statement of the amounts of fat, protein, sugar, and salts present. As a result of our somewhat detailed study of cow's milk, methods have been elaborated which allow of a more specific study of the milk constituents and enable us to form more definite opinions as to the condition in which they are present in milk. As these methods have been fully described in a previous paper<sup>1</sup> it will suffice to state here that the milk is first separated by means of a clay filter into two portions: the serum, which contains all the soluble constituents; and the unfilterable portion, which contains those substances which are present in the milk in the form of an emulsion or a colloid. These two portions are then studied separately.

### *Acidity of human milk.*

When the acidity is determined by the method recently published<sup>2</sup> it is found that the acidity of the serum of fresh milk is the same as that of the original whole milk, which proves that the serum contains all the acid bodies in the milk. It will be shown later that this acidity is in all probability due to acid phosphates and that from 3 to 6 cc. of tenth normal alkali are required to make 100 cc. of milk neutral to phenolphthalein.

<sup>1</sup> L. L. Van Slyke and A. W. Bosworth: this *Journal*, xx, p. 135, 1915; *New York Agricultural Experiment Station Technical Bulletins*, No. 39, 1915.

<sup>2</sup> Van Slyke and Bosworth: this *Journal*, xix, p. 73, 1914; *New York Agricultural Experiment Station Technical Bulletins*, No. 37, 1914.

In this connection it is well to call attention to the erroneous idea which has prevailed as to the difference in the acid reaction of human milk and cow's milk. This has been due to the incorrect method used to determine the acidity. The high acid figures previously obtained for cow's milk were due to the interference of the neutral calcium phosphate,  $\text{CaHPO}_4$ , which is present in cow's milk but not in human milk, as will be shown later on in this paper. By using the method mentioned above it is found that 100 cc. of cow's milk will require the addition of from 4.4 to 7.0 cc. of tenth normal alkali to make it neutral to phenolphthalein. The acidity of human milk and cow's milk is thus seen to be practically the same, and the practice of adding lime water to modified cow's milk used for infant feeding as a means of correcting the acidity is thus seen to have no foundation.

*The serum of human milk.*

The serum of fresh human milk resembles water in appearance. It is acid to phenolphthalein and alkaline to methyl orange, which would indicate that the acidity is due to acid phosphates. Its composition, in comparison with that of whole milk, is given in the following table.

TABLE I.  
*Composition of human milk and its serum.*

CONSTITUENTS	ORIGINAL MILK 100 cc.	MILK SERUM 100 cc.	MILK CONSTITUENTS IN SERUM
	gm.	gm.	per cent
Fat.....	3.30	0.00	0.00
Casein.....	*1.20	0.00	0.00
Albumin.....		0.131	* (13.10)
Nitrogen in other compounds calculated as protein.....	0.307	0.307	100.00
Citric acid.....	0.1055	0.1055	100.00
Phosphorus, organic.....	0.0008	0.00	0.00
Phosphorus, inorganic.....	0.0148	0.0148	100.00
Calcium.....	0.0354	0.0214	60.45
Magnesium.....	0.0030	0.0030	100.00
Sodium.....	0.0147	0.0147	100.00
Potassium.....	0.0711	0.0711	100.00
Chlorine.....	0.0375	0.0373	100.00

\* The determination of casein in the whole milk was very unsatisfactory, and for that reason the amount is not given in the table. It was about 0.2 gm. per 100 cc. of milk.

*The unfilterable portion of human milk.*

By reference to Table I it is seen that that portion of the milk which does not pass through the clay filter contains the fat, the casein, and part of the albumin and calcium. The fat can be removed by extraction with ether, leaving the protein and calcium. It was found impossible to separate the calcium from the protein by any mechanical means; so we conclude that it is in chemical combination with the protein. It is important to note that evidence seems to indicate that the albumin as well as the casein is combined with calcium. This will be investigated more carefully before any definite report is made.

The figures in Table I show that human milk, unlike cow's milk, contains no insoluble inorganic phosphates; *i.e.*, no dicalcium phosphate,  $\text{CaHPO}_4$ . This substance, if it were present, would be found in the portion of the milk which failed to pass through the clay filter.

*Principal compounds of human milk.*

As a result of our studies of human milk the following arrangement is offered as representing the probable condition in which the constituents are present in human milk of average composition.

	<i>per cent</i>
Fat.....	3.30
Milk sugar.....	6.50
Proteins combined with calcium.....	1.50
Calcium chloride.....	0.059
Mono-potassium phosphate ( $\text{KH}_2\text{PO}_4$ ).....	0.069
Sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ).....	0.055
Potassium citrate ( $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$ ).....	0.103
Mono-magnesium phosphate ( $\text{MgH}_4\text{P}_2\text{O}_6$ ).....	0.027

My thanks are due to Dr. Henry I. Bowditch, who has used funds at his disposal to pay for the milk used for this investigation.



1

## THE ESTIMATION OF FAT.

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(Received for publication, February 3, 1915.)

In the course of the nutrition investigations in this laboratory we have occasion to make a great many estimations of fat in samples of portions of animals and in feeding stuffs. The usual Soxhlet extraction of the thoroughly dried sample with ether is the method that has been employed. It is understood that this method gives a determination of the crude fat or total ether-soluble material. By the Soxhlet extraction of dried blood we have been unable to find any ether-soluble substances. It has been very difficult to obtain concordant results with such material as brain, liver, and bran, and it has been found that sixteen hours' extraction of these substances would not remove all the ether-soluble material. A re-extraction frequently adds a very appreciable amount of crude fat. It is because of these difficulties that we have investigated the methods proposed for accurate fat estimation in the hope that we could find a method that would give a true determination of the fat present, and at the same time permit application in a laboratory where a great many determinations must be made.

Kumagawa and Suto<sup>1</sup> have made a study of the general methods of fat estimation. The methods proposed by Rosenfeld, by Dormeyer, and by Glickin are also extraction methods, and therefore are no more accurate than the regular Soxhlet method. Kumagawa and Suto saponify the fat with 5N sodium hydroxide solution. They transfer the soap to a separatory funnel and acidify with a mineral acid. The fatty acids are shaken out with ether, the ethereal solution is evaporated to dryness, taken up with ether, and filtered through asbestos. The filtrate after being dried is taken up with petroleum ether and again filtered. The filtrate is evaporated and dried to constant weight. The residue is taken up with petroleum

<sup>1</sup> M. Kumagawa and K. Suto: *Biochem. Ztschr.*, viii, pp. 212-347, 1908.

ether and treated with alcoholic potash. The petroleum ether is removed and the alcoholic solution is again shaken with petroleum ether. These combined ether extracts are evaporated and taken up with a little alcoholic sodium hydroxide. The solution is evaporated, dried, extracted with petroleum ether, and filtered. The filtrate is evaporated and dried to constant weight. This weight of unsaponifiable material will give by subtraction the true amount of fatty acids which is reported as fat by multiplying by the factor 1.046. This method has been shown to give very satisfactory results on many substances, but has not worked satisfactorily with blood.

Shimidzu<sup>2</sup> has modified the above method for work with blood. He first extracts the blood with 95 per cent alcohol and filters through a cloth filter. The residue is again extracted with boiling alcohol. The combined alcoholic filtrates are saponified with 5N sodium hydroxide and the determination is continued as directed by Kumagawa-Suto. Following these directions we made several determinations of fat in cow's blood with the following results.

TABLE I.

*Blood.*

WEIGHT OF SAMPLE	WEIGHT OF FATTY ACIDS AND UNSAPO- NIFIABLE SUB- STANCES	WEIGHT OF UNSAPO- NIFIABLE SUB- STANCES	WEIGHT OF FATTY ACIDS	WEIGHT OF NEUTRAL FAT	NEUTRAL FAT	TIME ON WATER BATH
gm.	gm.	gm.	gm.	gm.	per cent	hrs.
20	0.1775	0.0813	0.0942	0.0985	0.493	3/4
20	0.1785	0.0935	0.0850	0.0889	0.445	3/4
20	0.1523	0.0967	0.0556	0.0582	0.291	6
10	0.0782	0.0471	0.0311	0.0325	0.325	3
9.5	0.0757	0.0353	0.0404	0.0423	0.444	4

Average of 5 determinations, 0.3996 per cent.

Greatest variation from average, 28 per cent.

Least variation from average, 11 per cent.

As can be seen from the above table the results are far from satisfactory. The great difference in the results may be due to several causes. The large number of filterings may cause a considerable loss. The great amount of cholesterol and other unsaponifiable substances in the blood may cause a difficulty in separating them from the fatty acids. Furthermore, it seems al-

<sup>2</sup> Y. Shimidzu: *ibid.*, xxviii, pp. 237-273, 1910.

most impossible to get rid of all the coloring matter. Even after six hours' drying, we were unable to obtain a colorless filtrate. Another probable source of error is the long drying on the water bath, which may cause some of the fatty acids to become insoluble in petroleum ether.<sup>3</sup>

In order to determine whether the large amount of cholesterol and other unsaponifiable substances in the blood soluble in petroleum ether has really a great deal to do with the fluctuation of results we determined the amount of fat in the liver of the same cow from which the blood was obtained, since it is stated by Paton<sup>4</sup> that the liver contains only about 0.03 to 0.05 per cent of cholesterol.

One hundred grams of liver were saponified and made up to volume, 1000 cc., with water; 100 cc. aliquots representing 10 grams of sample were then taken and analyzed.

TABLE II.

*Liver.*

WEIGHT OF SAMPLE	WEIGHT OF FATTY ACIDS AND UNSAPONIFI- FIABLE SUB- STANCES	WEIGHT OF UNSAPONIFI- ABLE SUB- STANCES	WEIGHT OF FATTY ACIDS	WEIGHT OF NEUTRAL FAT	NEUTRAL FAT
gm.	gm.	gm.	gm.	gm.	per cent
10	0.2125	0.0194	0.1931	0.2020	2.020
10	0.2205	0.0273	0.1934	0.2023	2.023

It is interesting to note how closely these two results agree, within 0.003 of a per cent. The amount of unsaponifiable substances in the liver soluble in petroleum ether is over 10 per cent of the total amount. This close agreement, it seems to us, would indicate that the less the amount of unsaponifiable substances, the less the errors introduced, regardless of the other errors enumerated above.

We next determined the fat in a sample of fresh, clear, back fat of pork. We weighed out 2.8262 grams and added 50 cc. of 95 per cent alcohol, and saponified it for three hours under a re-

<sup>3</sup> P. Hartley: *Jour. Physiol.*, xxxvi, p. 17, 1909; xxxviii, p. 353, 1909. It may be mentioned here that Bloor recently published a new nephelometric method for small amounts of fat in blood. *This Journal*, xvii, p. 377, 1914.

<sup>4</sup> D. N. Paton: *Jour. Physiol.*, xix, p. 191, 1895-96.



flux condenser. There was thorough saponification. The alcohol was boiled off as completely as possible. The solution was then made up to a liter of solution with water, and 100 cc. aliquots were taken. The petroleum ether extract was perfectly colorless.

TABLE III.

*Clear back fat of pork.*

WEIGHT OF SAMPLE	WEIGHT OF FATTY ACIDS AND UNSAPONIFI- FIABLE SUB- STANCES	WEIGHT OF UNSAPONIFI- ABLE SUB- STANCES	WEIGHT OF FATTY ACIDS	WEIGHT OF NEUTRAL FAT	NEUTRAL FAT
gm.	gm.	gm.	gm.	gm.	per cent
0.2826	0.2404	0.0010	0.2394	0.2504	88.62
0.2826	0.2356	0.0009	0.2347	0.2455	86.87

*Comparison of results by Soxhlet extraction.*

As mentioned before, no ether-soluble material was obtained by extraction of the dried blood. Twice extraction of the liver (after drying *in vacuo*) gave 2.723 per cent of ether-soluble material. The fresh sample of clear back fat of pork was analyzed, giving moisture 7.68 per cent, and fat (ether-soluble) 90.13 per cent. It will be noted that the results obtained by the Soxhlet extraction method and the Kumagawa-Suto method do not agree.

Lieberman and Székely<sup>5</sup> have proposed a method which consists in saponification of the fat, acidifying the soap with sulphuric acid. The acidified solution, in a specially graduated flask, is shaken with petroleum ether and the flask filled to the mark with a saturated sodium chloride solution. After thorough mixing an aliquot of the petroleum ether layer is taken, mixed with neutral alcohol, and titrated with standard alcoholic potash. Phenolphthalein is used as an indicator. The soap formed is evaporated to dryness, weighed, and the results are calculated to neutral glycerides.

In studying this method in comparison with the other methods, we tried some modifications which seemed to be advantageous, and propose the following method.

*Description of proposed method.* The sample is heated for two hours with 30 cc. of a 20 per cent sodium hydroxide solution.

<sup>5</sup> L. v. Lieberman and S. Székely: *Arch. f. d. ges. Physiol.*, lxxii, p. 360, 1898.

Place the beaker in the water bath and cover with a funnel having the stem cut off. During this saponification the mixture is stirred a few times.

The solution while still warm is transferred to a glass-stoppered separatory funnel of about 300 cc. capacity. The beaker is washed out two or three times with warm water. The solution is then acidified with 35 cc. of a 20 per cent hydrochloric acid solution (specific gravity 1.1). After thorough cooling the contents of the separatory funnel are shaken out with ether. The combined portions of the ether solution are filtered and evaporated to dryness on the water bath. The residue is next taken up with about 25 cc. of fat-free petroleum ether (boiling point 30°–50°C.), and about 10 or 15 cc. of 95 per cent alcohol are added. This is titrated with  $\frac{N}{10}$  alkali, using about two drops of a 1 per cent solution of phenolphthalein as indicator. The end-point is sharp and distinct.

With a sample of blood an alcohol extraction should first be made as recommended by Shimidzu.<sup>6</sup>

With a sample rich in fat we recommend that saponification be brought about in the presence of alcohol, using a reflux condenser to prevent loss of alcohol during boiling; after saponification the alcohol can be driven off by boiling.

The following tables give the determination of the fat in blood, liver, and clear back fat of pork by the proposed method.

TABLE IV.

*Blood.*

WEIGHT OF SAMPLE	NO. OF CC. OF 0.0499/ <i>N</i> NaOH	WEIGHT OF NaOH	WEIGHT OF FATTY ACIDS	WEIGHT OF NEUTRAL FAT	FAT
<i>gm.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
20	3.60	0.0072	0.0498	0.0521	0.260
20	3.50	0.0070	0.0484	0.0506	0.253
10	1.85	0.0037	0.0256	0.0268	0.268
9.5	1.78	0.0036	0.0246	0.0258	0.272

Average per cent, 0.263.

Greatest variation from average, 3.7 per cent.

Least variation from average, 0.95 per cent.

<sup>6</sup> Shimidzu: *loc. cit.*

This fat determination shows very concordant results, and although it differs from the average of five determinations of the same sample by the Shimidzu modification of the Kumagawa-Suto method, it will be noted that the greatest variation from the average is only 3.7 per cent, compared with a corresponding variation of 28 per cent in the results from the Kumagawa-Suto method.

TABLE V.  
*Liver.*

WEIGHT OF SAMPLE	NO. OF CC. OF 0.0499/N NaOH	WEIGHT OF NaOH	WEIGHT OF FATTY ACIDS	WEIGHT OF NEUTRAL FAT	FAT
gm.		gm.	gm.	gm.	per cent
10	13.45	0.0265	0.1834	0.1918	1.918
10	13.50	0.0270	0.1842	0.1927	1.927
10	14.50	0.0290	0.2005	0.2097	2.097

Average per cent, 1.981.

TABLE VI.  
*Clear back fat of pork.*

WEIGHT OF SAMPLE	NO. OF CC. OF 0.0499/N NaOH	WEIGHT OF NaOH	WEIGHT OF FATTY ACIDS	WEIGHT OF NEUTRAL FAT	FAT
gm.		gm.	gm.	gm.	per cent
0.2826	17.62	0.0352	0.2437	0.2549	90.18
0.2826	17.60	0.0351	0.2434	0.2546	90.08

Average per cent, 90.13.

TABLE VII.  
*Comparison of three methods.*

SUBSTANCE	METHOD	FAT
Blood.....	Soxhlet	0.000
"	Kumagawa-Suto	0.400
"	Proposed method	0.263
Liver.....	Soxhlet	2.722
"	Kumagawa-Suto	2.021
"	Proposed method	1.981
Back fat of pork.....	Soxhlet	90.13
" " " "	Kumagawa-Suto	87.74
" " " "	Proposed method	90.13

As can be seen from Table VII the Soxhlet method does not compare at all favorably except when working with almost pure fat. With the blood the ether did not extract anything, while with the liver it extracted 35 per cent of substances other than fat. This agrees with Paton (1895) who claimed that lecithin is a constant constituent of ether extract of liver. The Kumagawa-Suto method has many possibilities of error, as has been pointed out previously, and is time-consuming. The proposed method, on the other hand, is very short and by no means complicated. In titrating a molecular weight has to be assumed. Kumagawa and Suto, in working with fats of different animal organs, claim that in the main they consist of 70 per cent oleic acid, 20 per cent palmitic acid, and 10 per cent stearic acid. This proportion would give an average molecular weight of 277. The use of an arbitrary acid molecular weight of 277 cannot account for the difference in the fat in the blood from that found by the Kumagawa-Suto method, as the use of an oleic acid molecular weight of 282 would only raise the average per cent of fat in the blood to 0.270. Similarly, in converting the acid into neutral fat the use of the factor 1.046 can introduce no appreciable variation, as palmitic acid has a factor of 1.049, stearic a factor of 1.044, and oleic a factor of 1.045.

#### SUMMARY.

The estimation of fat by the use of solvent alone gives all substances soluble in the menstruum employed. Simple extraction therefore gives approximately true results for fat only when that is the only soluble substance present. In the back fat of the hog this is the case, and we obtained very concordant results with the Soxhlet method; but with blood and liver the results were not reliable. The Kumagawa-Suto method is difficult of manipulation, and there seem to be many possibilities of error in the separation of the unsaponifiable substances. With the method proposed this separation is avoided. The use of the arbitrary factor does not introduce an appreciable error.



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